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# Regulation of Cell Adhesion by the FERM Proteins, PTPN14 and Merlin

Patty Dimarco Hewitt

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REGULATION OF CELL ADHESION BY THE FERM  
PROTEINS, PTPN14 AND MERLIN

by

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REGULATION OF CELL ADHESION BY THE FERM  
PROTEINS, PTPN14 AND MERLIN

A  
DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

May 2015

This dissertation is dedicated to my parents:

Carl and Donna Dimarco

Their unparalleled love, support and sacrifice have grounded me in my faith and driven my educational pursuits.

This dissertation is also dedicated to my husband, Jonathan, and our two children, Trenton and Payton. This work was completed with their loving support, laughter and entertainment.



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# REGULATION OF CELL ADHESION BY THE FERM PROTEINS, PTPN14 AND MERLIN

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Cell-cell adhesion is critical for the control of tissue organization and homeostasis. A family of proteins that regulate cell-cell adhesions is the FERM (4.1 protein, Ezrin, Radixin, Moesin) domain-containing proteins. One FERM domain protein, the non-receptor tyrosine phosphatase PTPN14, is mutated or deleted in several human cancers suggesting that it may be involved in tumor development and/or progression. Additionally, the loss of the FERM domain protein Merlin is associated with tumor development and metastasis. Both PTPN14 and Merlin have been shown to localize and possibly regulate adherens junction (AJ) functions. This work sought to determine if PTPN14 is required for the establishment of epithelial adhesion and polarity. Using mammalian epithelial cells as a model to test these hypotheses, we down-regulated the tyrosine phosphatase, PTPN14. Decreased levels of PTPN14 increased E-cadherin membrane movement at cell contacts, disrupting cell contacts and abolishing E-cadherin localization during primordial lumen formation. In cells with established AJs and TJs, decreased levels of PTPN14 led to an increase in  $\beta$ -catenin Y654 phosphorylation, promoting increased membrane mobility of E-cadherin and subsequent destabilization of cell contacts. This was dependent on PTPN14 phosphatase activity at  $\beta$ -catenin Y654 opposing Src-mediated disruption of AJs. In cells that lacked PTPN14, for an extended time, I observed alterations in the morphology of the epithelial cells to a spindle morphology coupled with increased cell migration, transcriptional repression of E-cadherin and activation of Slug. Similar to the regulation of the AJ by PTPN14, we examined another FERM domain protein, Merlin, and

found that specific post-translational modifications in its unique N-terminal region regulate distinct AJ-mediated activities. Mutation of a single serine residue in the N-terminal region of Merlin blocked Merlin loading onto the AJ during early cell-cell adhesion formation. Disruption of Merlin AJ loading leads to actin disorganization and loss of contact inhibition of proliferation. Additionally, I found that phosphorylation of a second site in the N-terminal region of Merlin affects the release of the apical proteins, atypical protein kinase C (aPKC) and partitioning defective 3 (Par3), to the apical membrane for the development of functional TJs. Blocking this specific phosphorylation site disrupts the formation of functional polarized epithelia. From this, we see that distinct post-translational modifications of Merlin can alter its interaction with polarity proteins and thus Merlin function in regulating epithelial cell adhesion and polarity. Overall, these results indicate that mutation or post-translation regulation of FERM proteins could promote increased migration and invasion in cells altering normal epithelial development possibly leading to loss of tissue function and disease.

## TABLE OF CONTENTS

Approval Sheet .....	i
Title Page .....	ii
Dedication .....	iii
Acknowledgements .....	iv
Abstract .....	vi
Table of Contents .....	viii
List of Illustrations .....	xii
<b>Chapter 1. Introduction</b> .....	1
Epithelial cell polarity and adhesion .....	2
ERM proteins .....	5
The tyrosine phosphatase, PTPN14 .....	7
The <i>Nf2</i> tumor suppressor, Merlin .....	8
The polarity proteins, Par3 and aPKC .....	11
<b>Chapter 2. Materials and Methods</b> .....	15
Cell Culture .....	16
Western Blot and Immunoprecipitation (IP) .....	16
Plasmids .....	18
Quantitative reverse-transcription-PCR .....	20
<i>In vitro</i> kinase assay .....	21
Reverse Phase Protein Array (RPPA) .....	21
Immunofluorescence and Live-Cell Imaging .....	22

Image Processing and Analysis .....	23
Protein Structure Modeling .....	24
 <b>Chapter 3. Regulation of Lumen Formation and Cell Adhesion by the</b>	
<b>Tyrosine Phosphatase , PTPN14 .....</b>	<b>25</b>
Introduction .....	26
Results .....	28
PTPN14 localizes to cell contacts during epithelial junction	
development .....	28
PTPN14 is necessary for lumen formation and junction	
organization .....	32
PTPN14 depletion disrupts formation of functional epithelial cell	
junctions .....	32
PTPN14 regulates $\beta$ -catenin-Y654 phosphorylation .....	36
PTPN14 regulates membrane mobility of E-cadherin at cell	
contacts .....	37
PTPN14 stabilizes E-cadherin mobility by opposing Src kinase	
activity at $\beta$ -catenin-Y654 .....	43
PTPN14 activity is necessary for E-cadherin-mediated lumen	
formation .....	44
Discussion .....	51

## **Chapter 4. PTPN14 Promotes Mesenchymal Phenotypes by Destabilization**

<b>of the Adherens Junction .....</b>	<b>54</b>
Introduction .....	55
Results .....	58
Decreased PTPN14 induces mesenchymal features in epithelial cells .....	61
Cells with diminished PTPN14 activity express markers of EMT ...	62
Knockdown of PTPN14 promotes a specific EMT response over time .....	62
Decreased PTPN14 activity triggers Notch signaling .....	64
Discussion .....	71

## **Chapter 5. Defining the Function of the *Nf2* Tumor Suppressor Protein,**

<b>Merlin, in Coordinating Cell Adhesion and Polarity .....</b>	<b>73</b>
Introduction .....	74
Results .....	75
Merlin is required for the establishment of junctions in intestinal epithelial cells .....	75
The N-terminal region of Merlin is required for cell polarity and is phosphorylated by aPKC .....	78
aPKC regulates Merlin localization and function through post- translational modifications .....	80

Discussion .....	82
<b>Chapter 6. Conclusions and Future Directions .....</b>	<b>86</b>
Fine-tuning PTPN14 activity at the AJ .....	91
PTPN14 in disease initiation and progression .....	93
Merlin: Adhesion, polarity and tumor progression .....	96
Bibliography .....	102
Vita .....	146



## LIST OF ILLUSTRATIONS

### Figure

1	Schematic overview of adherens and tight junctions in simple columnar epithelia .....	4
2	Domain structure of ERM family members .....	6
3	Overview of junctional and polarity proteins .....	9
4	PTPN14 localizes to cell contacts during epithelial junction development ..	30
5	PTPN14 associates with the AJ and TJ proteins during junction development .....	31
6	PTPN14 is necessary for lumen formation and junction organization .....	33
7	PTPN14 depletion disrupts formation of functional epithelial cell junctions	35
8	PTPN14 regulates $\beta$ -catenin-Y654 phosphorylation .....	38
9	PTPN14 regulates membrane mobility of E-cadherin at cell contacts .....	40
10	Re-expression of full-length PTPN14 .....	42
11	PTPN14 stabilizes E-cadherin mobility by opposing Src kinase activity at $\beta$ -catenin-Y654 .....	45
12	$\beta$ -catenin protein expression and measurement of Src activity .....	47
13	Quantitation of E-cadherin bridge during early lumen formation .....	48
14	PTPN14 activity is necessary for E-cadherin-mediated lumen formation ...	50
15	Decreased PTPN14 alters epithelial cell morphology .....	59
16	Decreased PTPN14 induces mesenchymal features in epithelial cells .....	60
17	Cells with diminished PTPN14 activity express EMT markers .....	63
18	Knockdown of PTPN14 promotes a specific EMT response over time.....	65

## Figure

19	EMT in cells with decreased PTPN14 is independent of TGF $\beta$ and Hippo signaling .....	66
20	Reverse Phase Protein Array (RPPA) for Caco-2 cells of varying lengths of time with decreased PTPN14 activity .....	68
21	Decreased PTPN14 activity triggers an increase in Notch .....	70
22	Merlin is required for the establishment of AJs and TJs in Caco-2 cells ....	77
23	The N-terminal region of Merlin is required for apicobasal polarity and is phosphorylated by aPKC .....	79
24	aPKC regulates Merlin localization and function through post-translational modifications .....	81
25	Post-translational modification of the N-terminus of Merlin regulates its role in contact inhibition .....	83
26	PTPN14 is a major regulator of AJ-mediated adhesion .....	88
27	Structural modeling of PTPN14 CRC patient-derived mutations .....	94
28	Post-translational modification of the N-terminal region of Merlin by aPKC regulates its function in the establishment of junctions and apicobasal polarity .....	97

## **CHAPTER 1.**

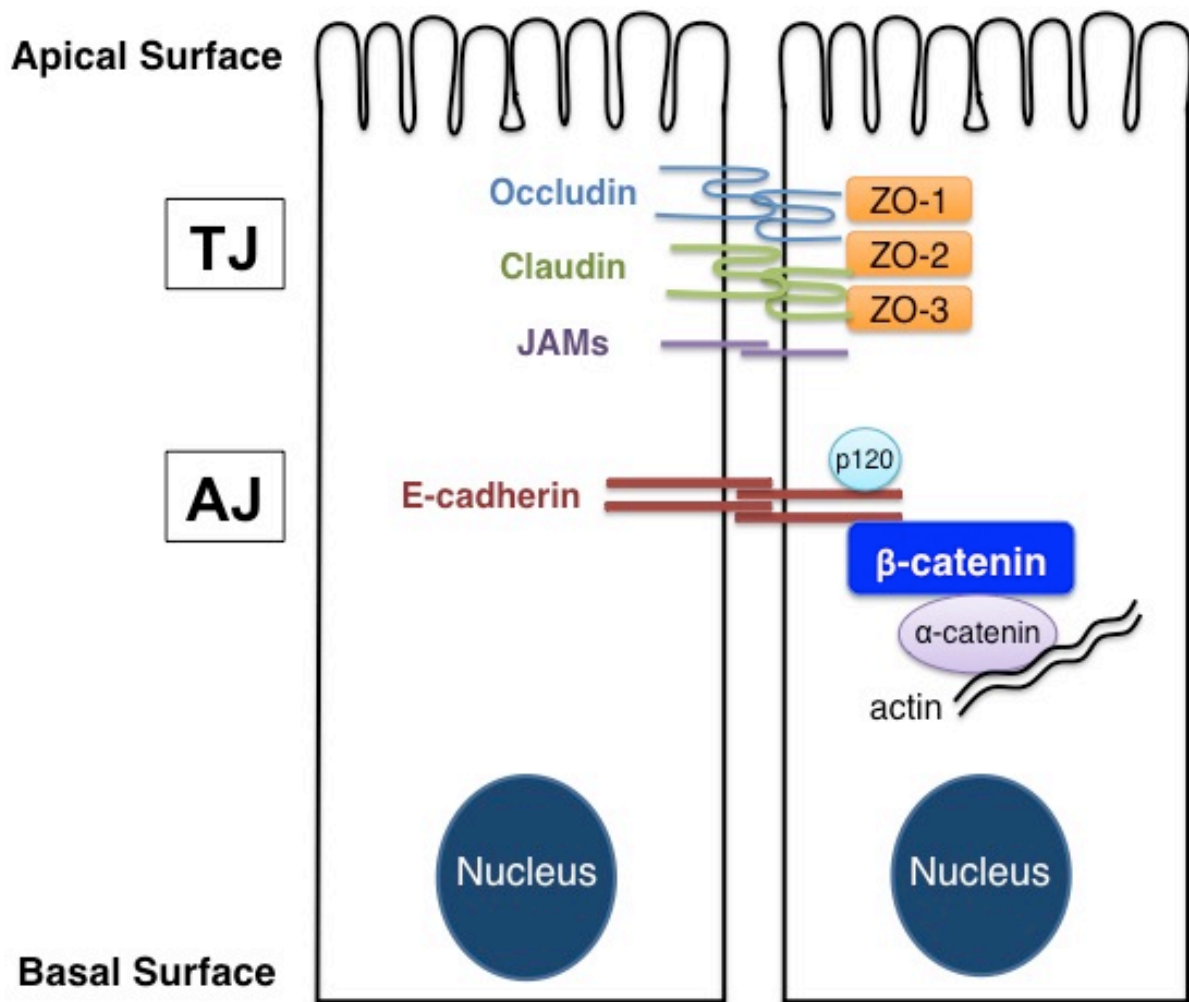
### **INTRODUCTION**

## **Epithelial cell polarity and adhesion**

Epithelia exist as sheets of adherent cells that create distinct physiological spaces in organisms<sup>1</sup>. The morphogenesis of epithelial tissue requires cell:cell interactions and cell polarity to shape organs and organisms<sup>2</sup>. For example, during neural tube formation, the neural plate and crest cells transition to a mesenchymal phenotype to induce movement and folding, which pulls in outer neural crest cells eventually forming a tube of cells that have switched adhesion functions which comprise the neural tube<sup>3</sup>. Development of tissues requires changes in both epithelial and mesenchymal-like functions and this can be regulated by epithelial to mesenchymal transition (EMT) where cells can switch from an epithelial state to a more mesenchymal state<sup>4,5 6-8</sup>. The reverse biological process, mesenchymal-epithelial transition (MET) occurs in specific tissue developments such as kidney development<sup>9-12</sup>. The adult kidney originates from two embryonic mesodermal derivatives, the ureteric bud and metanephric mesenchyme. In the mammalian embryo, metanephric mesenchyme is induced by the ureteric bud to undergo a sequence of morphogenetic events that transition the mesenchyme to an epithelium to ultimately produce most of the tubules of the mature nephron<sup>13</sup>. Interestingly, reports have postulated that due to similar morphological properties between primary tumors and metastatic lesions, cells may restore certain epithelial characteristics through MET at a metastatic site<sup>14,15</sup>. In many solid tumor types, cancer cells hijack EMT and MET to acquire more migratory and invasive characteristics and eventually establish themselves at secondary sites<sup>16,17</sup>. Importantly, many of the same molecular events that drive EMT during embryonic developmental are the same events that mediate EMT during tumor progression<sup>18</sup>; therefore, understanding the processes that regulate EMT has significant clinical relevance at both the developmental and disease level. During epithelial morphogenesis, the junctional structures

known as adherens and tight junctions (AJs and TJs, respectively) that mediate cell adhesion and apicobasal polarity are regulated by EMT<sup>19</sup>.

Epithelial tissues are highly organized structures that use cell adhesion and polarity to help form epithelial tissues. For example, when intestinal epithelia is fully formed, sheets of polarized columnar epithelia are organized with an apical region which faces the lumen or outer surface and a basolateral region that is in contact with the basal lamina<sup>20,21</sup> (Figure 1). Apical TJs, or zonula occludens, appear as a series of membrane appositions (or ‘kissing points’) that provide a continuous seal to facilitate adhesion between adjacent cells<sup>22</sup>. TJs have crucial functions by both serving as a barrier to the passage of molecules at cell:cell contacts and acting as a fence to maintain polarity by separating the apical and basolateral domains<sup>23-26</sup>. Over 40 TJ proteins have been identified including claudins<sup>27</sup>, occludins<sup>28</sup>, junctional adhesion molecules (JAMs)<sup>29</sup>, tricellulin<sup>30</sup>, zonulin<sup>31</sup> and numerous scaffolding proteins<sup>32-34</sup>. Of these, claudins connect to the actin cytoskeleton through interactions with ZO-1, -2 and -3, which are scaffolding proteins shown to play a critical role in the formation of TJs<sup>35</sup> and in the maintenance of barrier function<sup>36</sup>. Disruption of TJ function is affiliated with a variety of pathological conditions such as ulcerative colitis<sup>37</sup>, irritable bowel syndrome (IBS), edema, cancer and blood-borne metastasis<sup>34</sup>. While TJs are important for barrier and fence functions, their assembly is preceded by and dependent on the formation of the sub-apical AJ. The AJ is a dynamic calcium-dependent structure that provides mechanical attachments between adjacent cells by initiating and stabilizing cell:cell contacts and regulating the actin cytoskeleton<sup>38,39</sup>. The core structural component of the AJ is the cadherin-catenin complex that consist of E-cadherin and the p120,  $\alpha$ - and  $\beta$ -catenins. E-cadherin is a single-pass, transmembrane glycoprotein that forms homophilic interactions of epithelial AJ.

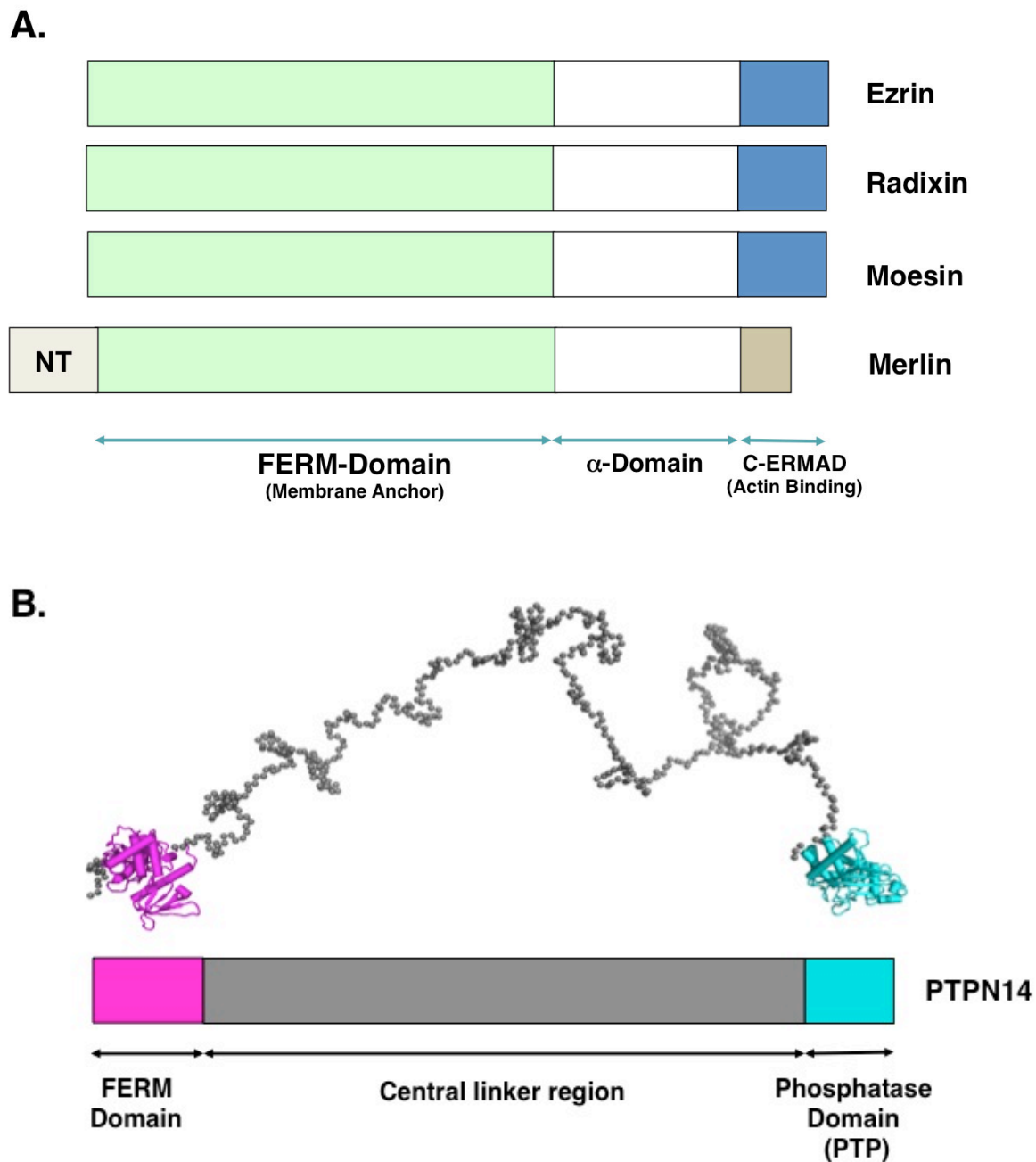


**Figure 1. Schematic overview of adherens and tight junctions in simple columnar epithelia.** The apical tight junctions (TJs) serve as a barrier for the passage of molecules at cell:cell contacts and are composed of the transmembrane proteins occludin, claudin and junctional adhesion molecules (JAMs). The scaffolding proteins, ZO-1, -2, and -3 link to transmembrane proteins and contribute to the TJ barrier function. The adherens junction (AJ) is just basal to the TJ and forms mechanical attachments between adjacent cells. At the core of the AJ is the cadherin-catenin complex that is composed of E-cadherin, p120-catenin and  $\beta$ -catenin.  $\beta$ -catenin associates with  $\alpha$ -catenin that connects to the actin cytoskeleton. E-cadherin must be reinforced to the actin cytoskeleton through the cytoplasmic cadherin-catenin complex at the AJ for cells to exhibit functional adhesion.

Classical cadherins, consisting also of N-, P- and R-cadherin<sup>40,41</sup>, have five characteristic extracellular cadherin repeat domains<sup>42</sup> that form trans-cadherin interactions between neighboring cells to initiate formation of the AJ<sup>43,44</sup>. Once AJ contacts are formed, cadherins initially cluster and then spread laterally to strengthen cell:cell contacts<sup>45</sup>. In the majority of epithelial cells, E-cadherin is connected to the actin cytoskeleton by its interaction with catenins<sup>45</sup> whose regulation can alter cell:cell contact strength and stability. The AJ interaction with the actin cytoskeleton is required in apical constriction during epithelial morphogenesis, tissue renewal and wound healing in multiple organisms<sup>42,44</sup>. Weakening of the AJ, by loss of E-cadherin or catenins, can alter other cell adhesions structures, including the TJ, which can compromise the overall integrity of cells by reducing cell adhesion<sup>46,47</sup>. Abnormal adhesion or loss thereof can lead to a host of chronic disease states that can progress to cancer, through aberrant signaling from the AJ<sup>41</sup>. In addition to the core AJ proteins, numerous other proteins interact with or localize near the AJ, helping to regulate cell adhesion and transmit signals from the cell surface to the actin cytoskeleton. One such group of proteins that link cytoskeletal signals to membrane proteins is the ERM (Ezrin, Radixin and Moesin) protein family<sup>48-51</sup>.

## **ERM proteins**

Originally named for its founding members Ezrin, Radixin and Moesin, ERM proteins are a highly evolutionarily conserved family of proteins important for regulating cell adhesion and membrane dynamics<sup>48</sup>. ERM proteins contain an amino-terminal FERM domain and a carboxy-terminal actin-binding domain separated by an extended alpha-helical linker domain<sup>52</sup> (Figure 2A). Through their FERM domain, ERM proteins can bind directly to adhesion molecules<sup>53,54</sup> or indirectly link to the plasma membrane<sup>55,56</sup>. A major structural property of



**Figure 2. Domain structure of ERM family members.** **A)** The ERM proteins are a highly evolutionarily conserved protein family and are named after the founding members, ezrin, radixin and moesin. ERM proteins contain an N-terminal membrane anchor FERM domain, an extended  $\alpha$ -helical domain and a C-terminal actin-binding domain. Merlin belongs to the ERM protein family although it lacks the C-terminal actin-binding domain. The first 17 amino acids at the N-terminal region (NT) of Merlin are unique among ERM molecules. **B)** PTPN14 contains an amino terminal FERM domain that makes it a close family member to the ERM proteins. The carboxy terminal catalytic phosphatase domain (PTP) is connected to the FERM domain via an unstructured central linker region. Protein model provided by Dr. Stefan Arold (KAUST).



ERM molecules is the ability to self-associate by a proposed intramolecular head-to-tail interaction between the amino- and carboxy-terminal domains<sup>52</sup>.

Although ERMs are believed to have overlapping functions, studies have uncovered critical roles for individual ERMs *in vivo*<sup>57-60</sup>. Of interest is the similar role for ERM proteins in establishing apical identity in intestinal lumen morphogenesis in *C. elegans* and mouse. The only *C. elegans* ERM orthologue, erm-1, and mouse Ezrin maintain the apical:junctional interface during early lumen morphogenesis by repositioning from the junctional region to the apical surface as polarity is established<sup>35,36</sup>. In a similar manner, the developing intestines undergo a switch in Ezrin distribution as cells differentiate and migrate up the villus axis<sup>61</sup>. Although ERM function is important for junctional remodeling during lumen morphogenesis, studies do not suggest that these proteins are required for AJ stability in established epithelia. Two proteins closely related to the ERM protein family that localize with and influence the AJ are the tyrosine phosphatase, PTPN14, and the tumor suppressor, Merlin.

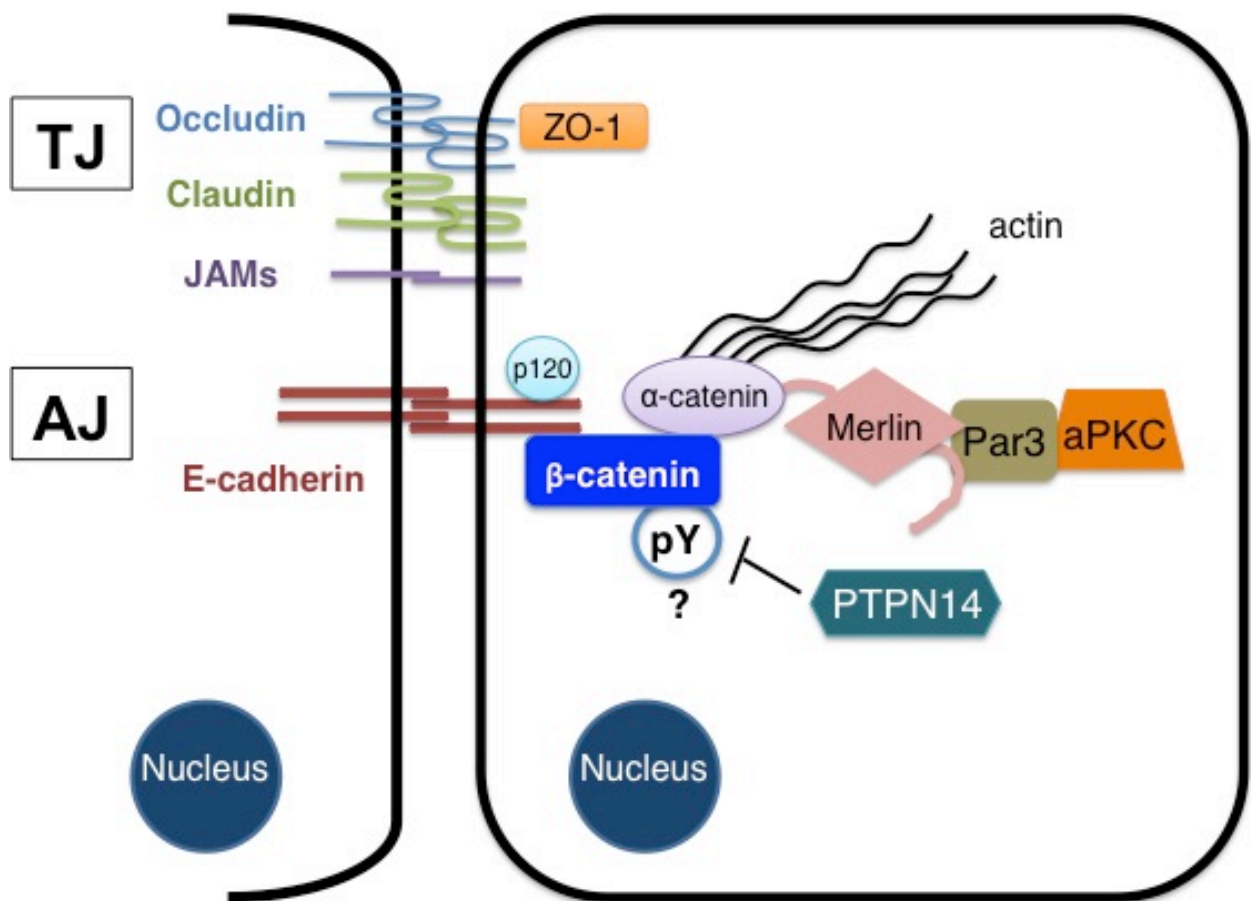
### **The tyrosine phosphatase, PTPN14**

The FERM-domain containing protein, PTPN14, also known as Pez, PTP36 and PTPD2, is a non-receptor tyrosine phosphatase that is expressed in multiple tissues<sup>62</sup>. PTPN14 is a member of the ERM protein family containing a N-terminal FERM domain; however, it is unique from other ERM family members because of its C-terminal phosphatase activity<sup>63,64</sup> (Figure 2B). Loss-of-function studies indicate a requirement for PTPN14 in the developing zebrafish heart and brain<sup>65</sup>. Interestingly, PTPN14 gain-of-function induces EMT and increases canonical transforming growth factor beta (TGF $\beta$ ) signaling in Madin-Darby Canine Kidney (MDCK) cells<sup>65</sup>, suggesting that PTPN14 may harbor tumor suppressor and oncogenic functions. *PTPN14* mutations occur in a subset of CRC patients, but mainly result in truncated proteins

with reduced phosphatase activity<sup>66</sup>. PTPN14 is shown to regulate tyrosine phosphorylation of the AJ protein,  $\beta$ -catenin, but the residue at which PTPN14 dephosphorylates  $\beta$ -catenin is unknown<sup>67</sup> (Figure 3). How and whether this activity impacts  $\beta$ -catenin signaling or AJ-mediated adhesion is not known. In addition, evidence suggests that PTPN14 also regulates p130 Crk-associated substrate (Cas) phosphorylation in CRC, which may influence the migratory and proliferative properties of these cells<sup>68</sup>. Recently PTPN14 has been implicated in the direct and indirect regulation of signaling pathways, including the TGF $\beta$  and Hippo signaling pathways<sup>65</sup>. The TGF $\beta$  pathway is known to induce EMT in a number of cell lines and tissues<sup>69,70</sup>. In addition to TGF $\beta$  signaling, PTPN14 directly interacts with and negatively regulates YAP, a core component of the Hippo signaling pathway, in a phosphatase-independent manner<sup>71-73</sup>. The Hippo pathway has also been implicated in inducing EMT and regulation of proliferation<sup>74</sup>.

### **The *Nf2* tumor suppressor, Merlin**

Mutations of the *neurofibromatosis type 2* (*NF2*) gene have been identified in multiple cancer types, including breast and liver cancer, malignant mesothelioma, sporadic meningiomas and schwannomas<sup>75-79</sup>. Merlin is the protein product of the *NF2* tumor suppressor gene and belongs to the ERM protein family. Merlin protein has a N-terminal FERM domain that is highly homologous to Ezrin but unlike Ezrin and other ERM family members, Merlin has an unique unstructured N-terminal region made up the first 17 amino acids and lacks the C-terminal actin-binding site common to ERM proteins<sup>80,81</sup> (Figure 2A). Despite the absence of this C-terminal actin-binding site, Merlin has been shown to be necessary to link membrane proteins and the actin cytoskeleton to establish stable AJs<sup>82-85</sup> (Figure 3). Homozygous deletion of Merlin in the



**Figure 3. Overview of junctional and polarity proteins.** The apical scaffolding protein, ZO-1, is localized at the tight junctions (TJs) in cells. The TJ is positioned just apical to the adherens junction (AJ) that is composed of a cadherin-catenin complex consisting of E-cadherin linked to  $\alpha$ -,  $\beta$ - and p120-catenins.  $\beta$ -catenin associates with  $\alpha$ -catenin that connects to the actin cytoskeleton. The FERM protein, PTPN14 dephosphorylates  $\beta$ -catenin at an unknown tyrosine residue. Merlin associates with  $\alpha$ -catenin via its N-terminal tail, linking it to the apical proteins, Par3 and aPKC.

developing mouse is embryonic lethal in mice<sup>86</sup> while heterozygous Merlin knockout mice (*Nf2*<sup>+/-</sup>) develop a series of highly metastatic tumors<sup>87</sup>. These data collectively suggest that Merlin may act as a tumor suppressor in multiple cell types. Numerous studies have uncovered three main mechanisms of Merlin tumor inhibition including contact-dependent growth inhibition<sup>85,88</sup>, decreased proliferation<sup>89-92</sup>, and increased apoptosis<sup>93</sup>, but how Merlin coordinates or separately regulates these mechanisms is not fully understood.

Merlin has a direct role in cell polarity by its restriction of apical Ezrin which guides proper spindle orientation<sup>94</sup> resulting in the formation of a single central lumen in epithelial cysts<sup>95-97</sup>. However, many uncertainties remain about the regulation of Merlin activity during cell junction formation and the establishment of apicobasal polarity. Recent studies address this by identifying that Merlin fails to associate with the AJ and does not localize to cell:cell contacts when it lacks the first 17 amino acids of its N-terminus (*Nf2*<sup>18-595</sup>)<sup>98</sup>. In addition, expression of *Nf2*<sup>18-595</sup> compromised the formation of functional TJs through Merlin's inability to associate with the AJ protein,  $\alpha$ -catenin<sup>84</sup>. Complete loss of Merlin also inhibits TJ formation and causes mis-localization of the polarity protein, atypical protein kinase C (aPKC)<sup>84</sup>. aPKC belongs to the PAR (Par3/Par4/aPKC) complex localized at the TJ in established epithelia<sup>99</sup>. Similarly, when aPKC kinase activity is eliminated, the formation of functional TJs is blocked and cell polarity is altered<sup>100</sup>, suggesting that mis-localization of aPKC affects cell membrane organization. Given the similar observations between loss of aPKC or loss of the N-terminal region of Merlin, it is possible that aPKC or another member of the PAR complex may play a role in regulating Merlin-mediated cell polarity and junction formation.

## **The polarity proteins, Par3 and aPKC**

Originally identified in a *C. elegans* screen for its importance in spatial restriction of the cytoskeleton and spindle asymmetry<sup>101</sup>, the PAR protein complex includes Par3, Par6, Cdc42, and the serine/threonine kinase aPKC<sup>102</sup>. Genetic studies in *Drosophila* established an important role for the PAR complex in the development and maintenance of polarity in epithelial cells<sup>103-105</sup>. Importantly, the PAR complex is required for proper polarity in mammalian cells<sup>106</sup>. Recent work has indicated that the PAR complex is a crucial signaling module that regulates polarization by integrating external and internal inputs to maintain homeostasis of normal cells. The complex components of the apical polarity complex are shown to be involved in tumor initiation and progression, both as regulators of oncogenic and tumor suppressor pathways, highlighting the importance of the PAR complex not only in tissue maintenance but also in inhibiting tumor formation<sup>107</sup>.

Par3 is a highly conserved polarity protein that has important roles in establishing apicobasal polarity<sup>108</sup>. When Par3 expression is lost, epithelial cells do not form electrically resistant TJs, resulting in “leaky” junctions<sup>109</sup>. How Par3 contributes to proper TJ formation is not known. Current reports suggest that during the early stages of junction formation, Merlin is essential for linking Par3 to the AJ<sup>84</sup>. After formation of the AJ, Par3 is localized to the apical domain for formation of TJs. How Par3 is transitioned from the AJ to the TJ remains undefined. In addition, what role Merlin has in the regulation of Par3 localization to the apical domain is unknown. One potential method of regulation could be through a role of Merlin in promoting the association of Par3 with aPKC.

Atypical protein kinases C is a member of the protein kinase C family of serine/threonine kinases whose activation is not dependent on diacylglycerol or calcium, but instead are controlled by the PAR protein complex<sup>110</sup>. Two *aPKC* genes have been identified in

mammals, *aPKC $\lambda$* <sup>111</sup> and *aPKC $\zeta$* <sup>112</sup>, which produce two different protein products that function as part of the PAR protein complex. To date, there is no evidence of the two isoforms having different functions in their roles as regulators of epithelial cell polarity, although their localization in the mouse epidermis is different suggesting that they may have spatially distinct mechanisms<sup>113</sup>. aPKC genomic amplification is seen in human cancers, including prostate, gastric, breast and ovarian cancers<sup>114,115</sup>. Interestingly, blocking aPKC kinase activity alters cell polarity and inhibits the formation of TJs, indicating that active aPKC kinase and Par3 localization are essential for proper TJ formation<sup>116</sup>. Furthermore, loss of Merlin causes mis-localization of aPKC away from the cell membrane, which could have an effect on cellular membrane organization<sup>84</sup>. These data suggest the existence of a key regulatory pathway in cell polarity and adhesion that may be coordinated by the *NF2* tumor suppressor protein, Merlin. Further understanding of the pathways that govern cell adhesion and polarity has the potential to uncover new therapeutic targets not only for epithelial tumors, but also for Schwann cell tumors that develop in *NF2* patients.

Epithelial cell polarity plays a vital role in a number of biological processes, such as cell adhesion<sup>117,118</sup>, division<sup>119-123</sup> and migration<sup>124-126</sup>. Defects in apicobasal polarity and adhesion are intimately linked to tissue disorganization resulting in tumorigenesis and ultimately, metastatic disease. The FERM domain protein family is widely known to play a role in regulating cell adhesion<sup>61,127,128</sup>. This thesis focuses on two FERM domain proteins, PTPN14 and Merlin, whose loss is observed in several human cancers.

The work in this thesis describes a new function that I have uncovered for a phosphatase-dependent role of PTPN14 in the regulation of AJ-mediated adhesion in intestinal lumen formation. Cells with decreased PTPN14 failed to form a lumen in 3D due to the inability to

form functional AJs and TJs. In cells with established AJs and TJs, decreased PTPN14 caused an increase in  $\beta$ -catenin-Y654 phosphorylation and E-cadherin membrane mobility leading to destabilization of cell contacts. My data demonstrates that PTPN14 phosphatase activity at  $\beta$ -catenin-Y654 opposes the Src kinase. Interestingly, when cells are exposed to decreased PTPN14 for an extended amount of time, the morphology of cells in culture changes from a classic epithelial cobblestone appearance to a spindle-cell morphology. This morphologic change is accompanied by loss of collective cell migration, loss of contact inhibition, transcriptional repression of E-cadherin and activation of Snail2/Slug potentially by Notch signaling from the AJ. This research highlights a novel role for PTPN14 as an important mediator of cell:cell adhesions and cell polarity through stabilization of essential epithelial features.

Additionally, the work described herein focuses on the step-wise post-translational modification of the FERM domain protein and tumor suppressor, Merlin, by aPKC to facilitate establishment of epithelial junctions and apicobasal polarity. When a single serine phosphorylation of the extreme N-terminal region of Merlin is blocked, Merlin is not loaded to the AJ during junctional development causing actin disorganization and loss of contact inhibition of proliferation. A subsequent phosphorylation event releases the polarity proteins, aPKC and Par3, from the AJ to the apical membrane for formation of functional TJs and proper epithelial polarity. This illustrates how post-translational modifications of Merlin can alter its protein function and interaction with polarity proteins to promote Merlin-mediated regulation of epithelial adhesion and polarity coordination.

In summary, my work shows the importance of FERM domain proteins in the development of functional epithelial cell adhesion and in the establishment of apicobasal polarity. My observations also indicate a critical role for FERM domain proteins in the maintenance of tissues to avoid alterations of epithelial cell adhesion that lead to tumor initiation and/or

progression. Cell adhesion is critical to maintain a protective epithelial barrier from bacterial and microbial infiltration. Consequently, altered cell adhesion can cause barrier defects that lead to inflammatory responses that promote tumor growth<sup>129</sup>. Therefore, increasing the understanding of the roles of PTPN14 and Merlin in controlling the establishment of cell adhesion and the maintenance thereof could open up future avenues for therapeutics in a clinical setting.



**CHAPTER 2.**  
MATERIALS AND METHODS

## Cell Culture

Human Caco-2<sup>BBE</sup> cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum (FBS). Production of lentiviral expression vectors used for targeted knockdown (pLKO.1) or over-expression (pCDH) was performed by transfecting 293T cells with the respective construct and the packaging vectors,  $\Delta$ VPR and VSVG. Twenty-hours after lentiviral infection, cells were selected with puromycin (pLKO.1) and/or hygromycin (pCDH).

For 3D culture, following trypsin treatment, cells were resuspended in media and passed through a 40 $\mu$ m-filter to obtain single-cell suspension. Cells were spun down for 5 minutes at 4<sup>o</sup>, then resuspended in 250 $\mu$ l of matrigel/collagen media (5% Matrigel, 0.02M Hepes, 1mg/ml Collagen 1) to a final concentration of 4x10<sup>4</sup> cells per milliliter. Cell suspensions were plated in an 8-well chamber slide, allowed to solidify for 30 minutes at 37<sup>o</sup>C, and then topped with media. Cells were re-fed after two days then collected on day six. The pharmacological inhibitor, Dasatinib, was added directly to the media at a concentration of 150nM two times per day.

The hanging drop assay was performed as previously described<sup>84,130</sup>. Caco-2 cells were trypsinized to a single cell suspension using a 40 $\mu$ m filter. Cells were collected at a density of 4x10<sup>6</sup> cells, spun down and resuspended in 2mL DMEM/10% FBS. 50 $\mu$ l drops of each cell type were hung on the bottom of a 35mM dish and placed in a 37<sup>o</sup>C incubator. Drops were pipetted 25x at one-hour intervals for three hours and then counted for 1-2 cells/clump, 3-6 cells/clump or 7+ cells/clump.

## Western Blot and Immunoprecipitation (IP)

For Western blots, cells were lysed using RIPA lysis buffer (50 mM Tris at pH 7.4, 1% Triton X-100, 1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA,

1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM sodium fluoride, 1 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin) followed by a brief sonication. Cell debris was cleared by centrifugation. Lysates were quantified by Bradford assay, separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked in 5% milk for 1 hour and incubated with primary antibody overnight at 4°C diluted in 1% milk.

For IPs, cells were lysed in Triton lysis buffer (1% Triton X-100, 50mM Tris-HCl pH 7.4, 140mM NaCl, 1mM EDTA, 1mM EGTA, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM sodium fluoride, 1 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin), followed by one round of sonication. Cell debris was cleared by centrifugation. Lysates were quantified using the Bradford assay. Protein lysates were diluted in Tween 20 buffer (50mM HEPES pH 8, 150mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM sodium fluoride, 1 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin) and incubated with Protein A sepharose beads then incubated with primary antibodies overnight at 4°C diluted in 1% milk. The precipitated complexes were washed with Tween 20 buffer 4 times then resuspended in protein loading buffer, boiled and loaded onto SDS-PAGE gels.

Primary antibodies used for Western blot, IP and/or immunofluorescence (IF) in this work were: Pez/PTPN14 (1:500 for IF and 1:750 for Western blotting, G-20, Santa Cruz), ZO-1 (1:500 for IF and Western blotting, 617300, Invitrogen), E-cadherin (1:1000 for IF and Western blotting, 610182, BD Biosciences),  $\alpha$ -catenin (1:1000 for Western blotting, 610193, BD Biosciences), p120 (1:2000 for Western blotting, 610133, BD Biosciences),  $\beta$ -catenin (1:2000 for IF and Western blotting, 610153, BD Biosciences),  $\beta$ -catenin (Tyr-654), phospho-specific (1:500 for IF and Western blotting, CP4021, ECM Biosciences),  $\beta$ -catenin (Tyr-489), phospho-specific (1:500 for Western blotting, CP2961, ECM Biosciences),  $\beta$ -catenin (Tyr-142), phospho-specific (1:500 for IF and Western blotting, CP1081, ECM Biosciences), Ezrin (1:100 for IF,

357300, Invitrogen), c-Myc (1:250 for Western blotting, SC-40, Santa Cruz), NF2/Merlin, C-terminal (1:1000 for IF and Western blotting, C-18, Santa Cruz), NF2/Merlin, N-terminal (1:1000 for IF and Western blotting, A-19, Santa Cruz), ZEB-1 (1:1000 for Western blotting, H-102, Santa Cruz), Snail2/Slug (1:500 for Western blotting, C19G7, Cell Signaling), Vimentin (1:1000 for Western blotting, 3932, Cell Signaling), Smad4 (1:500 for IF, 9515, Cell Signaling), total-YAP (1:1000 for IF, 4912, Cell Signaling),  $\beta$ -tubulin (1:20,000 for Western blotting, T7816, Sigma). For IF, filamentous actin was used to label Phalloidin (1:200, Alexa Fluor 488 and 568, Invitrogen) and nuclei were labeled with 4'6'-diamidino-2-phenylindole (DAPI).

## Plasmids

pLKO.1 lentiviral plasmids targeting human *PTPN14* were obtained from Open Biosystems (Clone ID NM\_005401.3-3772s1c1 and NM\_005401.3-754s1c1). The human *PTPN14* cDNA was ordered from Thermo Scientific (Clone ID 8143806). The p-GEM-T cloning vector was used to PCR out the human *PTPN14* fragment into the pCDH-CMV-MCS-EF1 lentiviral mammalian expression vector (System Biosciences). sh-RNA resistance was conferred by making six silent mutations in the respective shRNA targeting sequence. The following QuickChange primers were used to mutate C1121 to M and make the *PTPN14* catalytic-dead mutant: 5'-CATCGTGTCCACTCTAGTGCTGGGGTGG-3' (forward primer) and 5'-CCACCCCAGCACTAGAGTGGACCACGATG-3' (reverse complement). The following QuickChange primers were used to mutate Y654 to F and make the non-phosphorylatable  $\beta$ -catenin mutant: 5'-GGAATGAAGGCGTGGCAACATTCGCAGCTGCTG-3' (forward primer) and 5'-CAGCAGCTGCGAATGTTGCCACGCCTTCATTCC-3' (reverse complement). N-terminal Myc epitope tags were added to  $\beta$ -catenin<sup>WT</sup> and  $\beta$ -catenin<sup>Y654F</sup> by

cloning into the pJ3M vector. All constructs were subcloned into the pCDH-CMV-MCS-EF1 mammalian lentiviral expression vector (System Biosciences).

The human *Nf2* shRNA targeting sequence 5'-AGGAAGCAGCCCAAGACATTC-3' was inserted into the pLKO.1 lentiviral plasmid. The pCDNA3 cloning vector was used to PCR out the human *Nf2* full-length fragment into the pCDH-CMV-MCS-EF1 lentiviral mammalian expression vector (System Biosciences). sh-RNA resistance was conferred by making three silent mutations in the respective shRNA targeting sequence. The following QuickChange primers were used to mutate S7 to A to make the Merlin non-phosphorylatable mutant: 5'-CCGGAGCCATCGCTGCTCGCATGAGCTTC-3' (forward primer) and 5'-GAAGCTCATGCGAGCAGCGATGGCTCCGG-3' (reverse complement). The following QuickChange primers were used to mutate S10 to A to make the Merlin non-phosphorylatable mutant: 5'-CCATCGCTTCTCGCATGGCCTTCAGCTCACTCAAGA-3' (forward primer) and 5'-TCTTGAGTGAGCTGAAGGCCATGCGAGAAGCGATGG-3' (reverse complement). The following QuickChange primers were used to mutate S12 to A and make the Merlin non-phosphorylatable mutant: 5'-CTTCTCGCATGAGCTTCGCCTCACTCAAGAGGAAGC-3' (forward primer) and 5'-GCTTCCTCTTGAGTGAGGCGAAGCTCATGCGAGAAG-3' (reverse complement). The following QuickChange primers were used to mutate S13 to A and make the Merlin non-phosphorylatable mutant: 5'-GCATGAGCTTCAGCGCCCTCAAGAGGAAGCAG-3' (forward primer) and 5'-CTGCTTCCTCTTGAGGGCGCTGAAGCTCATGC-3' (reverse complement). The S7/10/12/13 to A Merlin non-phosphorylatable mutant was generated in a stepwise process using the QuickChange primers for each S to A mutation. The pFlex cloning vector was used to add an N-terminal flag tags to the *Nf2* fragment.

## Quantitative reverse-transcription-PCR

Total RNA was isolated from cells using Trizol followed by chloroform extraction and 2µg RNA was used for first strand cDNA synthesis using the SuperScript® First-Strand Synthesis System (Invitrogen). The two primer sequence sets for human PTPN14 were 5'-ATGCCTTTTGGTCTGAAGCTC-3' (forward primer 1), 5'-CCCTGTGCTTCCACCGAC-3' (reverse complement 1), 5'-AGCTGCGAGAGACGCACTA-3' (forward primer 2) and 5'-GCTCATTAGCGAATTTGTCCAGA-3' (reverse complement 2). The two primer sequence sets used for human E-cadherin were 5'-CGAGAGCTACACGTTACGG-3' (forward primer 1), 5'-GGGTGTCGAGGGAAAAATAGG-3' (reverse complement 1), 5'-ATTTTTCCTCGACACCCGAT-3' (forward primer 2), and 5'-TCCCAGGCGTAGACCAAGA-3' (reverse complement 2). The two primer sequence sets for human CDH2/N-cadherin were 5'-TCAGGCGTCTGTAGAGGCTT-3' (forward primer 1), 5'-ATGCACATCCTTCGATAAGACTG-3' (reverse complement 1), 5'-AGCCAACCTTAAGTGGAGGAGT-3' (forward primer 2) and 5'-GGCAAGTTGATTGGAGGGATG-3' (reverse complement 2). The primer sequences used for human Snail1 were 5'-TCGGAAGCCTAACTACAGCGA-3' (forward primer) and 5'-AGATGAGCATTGGCAGCGAG-3' (reverse complement). The primer sequences used for human Snail2/Slug were 5'-CGAACTGGACACACATACAGTG-3' (forward primer) and 5'-CTGAGGATCTCTGGTTGTGGT-3' (reverse complement). The primer sequences used for human BIRC3 were 5'-AGAGAGGAGCAGAGATGGAGCA-3' (forward primer) and 5'-TTTGTTCCTCCGCATTAGTGC-3' (reverse complement). The primer sequences used for human CTGF1 were 5'-GGGCCTCTTCTGCGATTTC-3' (forward primer) and 5'-ATCCAGGCAAGTGCATTGGTA-3' (reverse complement). The primer sequences used for human AREG were 5'-GTGGTGCTGTCGCTCTTGATA-3' (forward primer) and 5'-

CCCCAGAAAATGGTTCACGCT-3' (reverse complement). The primer sequences used for mammalian GAPDH were 5'-ACAACCTTTGGTATCGTGGAAGG-3' (forward primer) and 5'-GCCATCACGCCACAGTTTC-3' (reverse complement). PCR was performed using the 7900HT Sequence Detection System (Applied Biosystems) with SYBR green quantification. Gene expression was normalized to *GAPDH* expression levels.

### ***In vitro* kinase assay**

100 ng of GST-aPKC- $\zeta$  (Echelon Biosciences) was mixed with Flag-Merlin or Flag-Ezrin produced in Sf9 cells or NT-Merlin, NT-Merlin 12/13A, NT-Merlin 7/10/12/13A produced by IVTT in kinase assay buffer (25 mM MOPS, pH 7.2, 12.5 mM  $\beta$ -glycerol-phosphate, 25 mM  $MgCl_2$ , 5 mM EGTA, 2 mM EDTA and 0.25 mM DTT). Following mixing on ice 0.25 mM of ATP and 10 mCi gamma- $^{32}P$ -ATP was added to the kinase reaction and incubated at 30°C for 20 minutes with occasional mixing. The reaction was stopped by addition of 6x SDS sample buffer and then boiled for 3 minutes. The kinase reaction was loaded on a SDS-PAGE gel and following electrophoresis the gel was stained with Coomassie, destained, dried and exposed to film.

### **Reverse Phase Protein Array (RPPA)**

Cells were lysed using the following lysis buffer: 1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM  $MgCl_2$ , 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM  $Na_3VO_4$ , 10% glycerol, 1 mM PMSF, 1 mM  $Na_3VO_4$ , 1 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin. Cell debris was cleared by centrifugation. Lysates were quantified by Bradford Assay and mixed with 4 x SDS sample buffer without bromophenol blue (40% Glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8. with 2-mercaptoethanol at 1/10 of the volume)

with a final mixture of 3 parts of cell lysate mixed with one part of 4 x SDS sample buffer. Duplicate control (scr), early (93C), middle (93B) and late (93A) PTPN14 depleted cells were submitted to the MD Anderson RPPA Core Facility and were stained for 285 unique antibodies and analyzed on Array-Pro then by supercurve Rx64 3.1.1. Samples were probed with the antibodies using a CSA amplification approach and visualized by DAB colorimetric reaction. The heatmap was generated in Cluster 3.0 as a hierarchical cluster using Pearson Correlation and shows overall patterns of protein levels.

### **Immunofluorescence and Live-Cell Imaging**

In 3D culture, Caco-2 cells were fixed in 3.7% Formalin in cytoskeletal buffer (CB) [10mM 2-(N-morpholino)-ethanesulfonic acid sodium salt (MES) at pH 6.1, 138 mM KCl, 3mM MgCl<sub>2</sub>, 2mM EGTA] for 20 minutes at room temperature, briefly washed in PBS and permeabilized in 0.5% Triton/PBS for 20 minutes at room temperature. After rinsing with PBS, cells were washed with 100mM Glycine/PBS for 3X15 minutes and blocked for one hour in PBS-T/0.2% Triton/1% BSA/10% goat serum. Primary antibodies were incubated overnight in blocking buffer at 4°C. After a brief wash with PBS-T, cells were incubated in secondary antibodies for one hour at room temperature. In 2D culture, Caco-2 cells were grown on glass coverslips to confluence. Cells were fixed with ice-cold methanol-acetone for 10 minutes at -20°C and then treated with 1.5N HCl for 10 minutes at room temperature. After blocking with PBS-T/10% HISS for 30 minutes, cells were incubated with primary antibody overnight at 4°C. After washing with PBS-T, cells were incubated with secondary antibodies diluted in PBST/10% HISS for 1 hour. All labeled cells were visualized with a Nikon A1 laser scanning confocal microscope and processed using the Nikon-Elements software (Nikon). 3D images were



deconvolved to remove excess background with the AutoQuant X3 software (Media Cybernetics).

For live-cell imaging and FRAP, Caco-2<sup>BBE</sup> cells were grown on a one compartment 35mm glass-bottom dish to confluence in DMEM supplemented with 10% FBS then switched to DMEM with no phenol red supplemented with 10% FBS before imaging. The cells were visualized using a Perkin Elmer spinning disc confocal microscope fitted with a Hamamatsu EMCCD 9100-13 camera and a 60X water objective. An attached control chamber allowed for temperature and CO<sub>2</sub> maintenance to preserve the cells in culture. Improvion Volocity analysis software (Perkin Elmer) was used to acquire live-cell imaging and FRAP data. Z-stacks were acquired every thirty seconds for five minutes. Imaging configuration was optimized for high fluorescence intensity per pixel. For FRAP acquisition, a region of interest (ROI) was selected by drawing a circular region of the same size at a single cell:cell contact and around a region in the background as a control. To provide steady-state fluorescence intensity, a minimum of three pre-bleach images at approximately one frame per second was taken before photo-bleaching. Laser intensity was set at 100% for the photo-bleaching event. Once photo bleaching occurred, multiple time points were obtained during the early stages of recovery and each FRAP recovery curve was collected for 10 minutes post-bleach. The FRAP curves were fitted with one-phase exponential equations. To remove excess background, live-cell imaging data was deconvolved using the AutoQuant X3 software (Media Cybernetics).

### **Image Processing and Analysis**

Live-cell imaging snapshots presented in figures 9 and 10 were processed using Bitplane's Imaris software. Individual GFP spots were tracked and each was less than 0.3 $\mu$ m in size and traveled a distance of no more than 5 $\mu$ m. Quantitative analysis of xyz planes were

acquired through cropping a single cell:cell contact in 3D and using the mean values for the horizontal ( $x$ ) plane  $\pm$  standard deviation. Five separate images were analyzed per cell type and representative images are shown in figures 9 and 10.

### **Protein Structure Modeling**

Structures were analyzed based on the PTPN14 PTP domain crystal structure (PDB id 2BZL). Loops missing in the experimental structure and point mutants were modeled using SwissModel. The PTPN14 FERM domain and the E79D mutant were produced using SwissModel, based on the FERM domain of merlin (28% sequence identity). Pymol was used for structural visualization and analysis. All structural modeling was performed by Dr. Stefan Arold (KAUST).

### **CHAPTER 3.**

#### **REGULATION OF LUMEN FORMATION AND CELL ADHESION BY THE TYROSINE PHOSPHATASE, PTPN14**

## Introduction

Organization of epithelial tissues requires controlled communication between neighboring cells to coordinate tissue development and morphogenesis<sup>131</sup>. Cells utilize numerous signaling pathways to interact with neighboring cells and to respond to external cues and changes in the extracellular matrix<sup>132</sup>. A primary means of direct cell communication is through specialized cell:cell contacts which aid in organizing cells in a polarized fashion. Cell polarization gives rise to accurate spatiotemporal responses to signals that come from neighboring cells and the surrounding microenvironment<sup>133</sup>. The formation of polarized cell layers largely depends on cell adhesion and the polarity complexes known as adherens and tight junctions (AJ and TJ, respectively). The AJ is a dynamic calcium-dependent structure that promotes the physical association of adjacent cells' cytoskeletons<sup>134,135</sup>. At the core of the epithelial AJ, E-cadherin acts as the main adhesive transmembrane protein<sup>136-138</sup>. E-cadherin binds directly to  $\beta$ -catenin, a linker to  $\alpha$ -catenin that binds actin filaments thereby connecting cadherins to the actin cytoskeleton<sup>139-144</sup>. The AJ interaction with the actin cytoskeleton is critical for regulating mechanical cues that drive cell polarity, directionality of cell migration and speed of migration during collective cell migration<sup>46,145</sup>. Proper collective cell migration is necessary for morphogenesis, tissue renewal and wound healing in multiple organisms<sup>46,47,146-148</sup>. Consequently, weakening of the AJ by down regulation of E-cadherin or deletion/mutation of the catenins, can compromise the formation of other cell adhesion structures, including the TJ, and establishment of cell polarity<sup>84</sup>. Interference of the coordinated formation of cell contacts and polarity can disrupt overall cellular integrity leading to loss of tissue function and disease<sup>46,47</sup>.

Cadherin-mediated adhesion is regulated on numerous levels throughout the cell. E-cadherin and  $\beta$ -catenin initially associate in the endoplasmic reticulum, and this complex is then trafficked to the plasma membrane<sup>149</sup>. Initial binding of E-cadherin extracellular domains on

neighboring cells promotes assembly of the E-cadherin and  $\beta$ -catenin complex with  $\alpha$ -catenin, linking this primordial AJ to the actin cytoskeleton<sup>150,151</sup>. Association with the actin cytoskeleton partially immobilizes the AJ which is thought to aid in local AJ complex clustering, in turn strengthening the AJ- mediated adhesion between adjacent cells<sup>152-155</sup>. Cadherin-mediated adhesion can also be regulated directly by phosphorylation of both the cadherins and catenins<sup>156</sup>. Tyrosine phosphorylation of E-cadherin alters its binding affinity for p120- and  $\beta$ -catenin in endothelial cells<sup>157</sup>.

Serine/threonine phosphorylation of p120-catenin protects E-cadherin from endocytosis, subsequently stabilizing the AJ<sup>158</sup>. Tyrosine phosphorylation of  $\beta$ -catenin can alter cell adhesion through disruption of  $\beta$ -catenin- $\alpha$ -catenin binding or by altering the adhesion properties of the E-cadherin/ $\beta$ -catenin complex. One such tyrosine kinase that regulates E-cadherin-mediated adhesion at the AJ is Src<sup>159,160</sup>. The pp60<sup>c-src</sup> protein kinase phosphorylates  $\beta$ -catenin at Y86 and Y654, but only phosphorylation at Y654 alters E-cadherin-mediated adhesion<sup>161</sup>. The c-Src proto-oncogene is connected to the initiation and progression of cancer in numerous tissues including breast, lung, colon, pancreas and skin<sup>162</sup>. Although much work has been done to extend our knowledge of the Src family of kinases in regulating the AJ in disease and cancer, relatively little is known about the opposing phosphatases that regulate Src-mediated tyrosine phosphorylation at the AJ.

PTPN14, also known as Pez, PTP36 and PTPD2, is a non-receptor tyrosine phosphatase<sup>64</sup> that is expressed in multiple tissues<sup>65</sup> and is significantly downregulated in colorectal cancer (CRC) (Oncomine microarray data). *PTPN14* mutations are found in a subset of CRC patients with the majority of these mutations resulting in truncated proteins with reduced phosphatase activity<sup>66</sup>. PTPN14 belongs to the FERM (Band 4.1, Ezrin, Radixin and Moesin) domain family of protein tyrosine phosphatases (PTPs) and is characterized by the conserved N-terminal FERM

domain and a C-terminal PTP domain divided by a flexible linker region<sup>63,64</sup>. The FERM domain family of proteins coordinates and regulates extracellular signals from the membrane to the cellular cytoskeleton<sup>127</sup>. PTPN14 is thought to regulate tyrosine phosphorylation of the AJ protein,  $\beta$ -catenin; however, the specific tyrosine phosphorylation site(s) regulated by PTPN14 are not known<sup>67</sup>. How and whether PTPN14 activity on  $\beta$ -catenin influences  $\beta$ -catenin signaling or AJ-mediated adhesion and tissue morphogenesis remains elusive. Considering the down regulation and/or mutation of PTPN14 in human disease as well as its possible role at the AJ, we examined the endogenous function of PTPN14 in polarized intestinal epithelial cells. Here we describe a mechanism of regulation of adhesion and apicobasal polarity by the protein tyrosine phosphatase, PTPN14, and investigate its endogenous function in stabilization of the E-cadherin/ $\beta$ -catenin complex at the AJs.

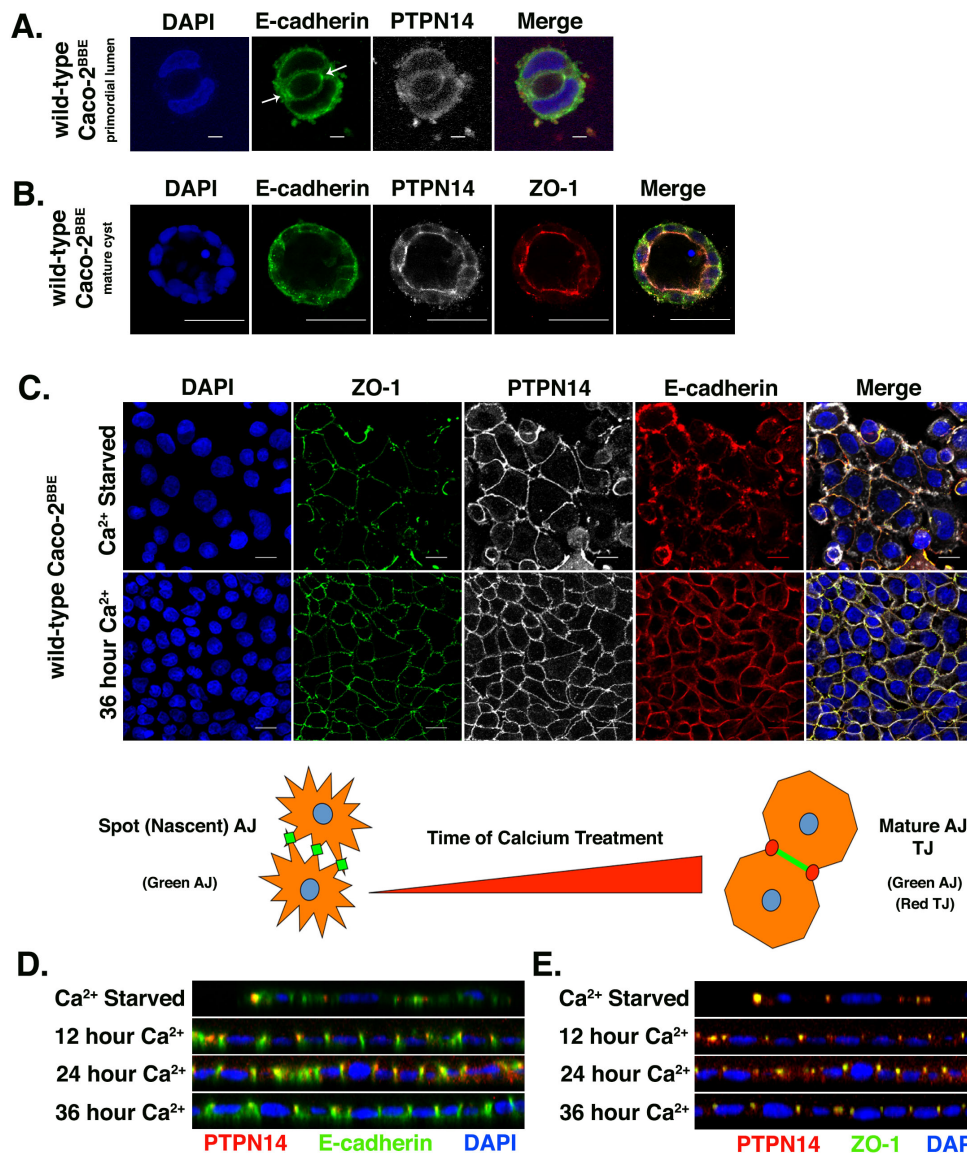
## **Results**

### **PTPN14 localizes to cell contacts during epithelial junction development**

Previous work revealed a capacity for PTPN14 to regulate both proliferation and migration pathways, but the mechanism for this activity has remained unclear<sup>65,67,72,163</sup>. To examine endogenous function(s) of PTPN14, we first sought to determine the localization pattern of PTPN14 in the well-established Caco-2<sup>BBE</sup> (hereafter referred to as Caco-2) intestinal epithelial cell line<sup>94</sup>. Caco-2 cells are a well-documented system to study apicobasal polarity during epithelial cyst formation<sup>94,95</sup>. To determine the localization of PTPN14 during lumen formation, we performed 3D lumen formation assays. When the primordial lumen is forming at the 2-cell stage, PTPN14 lines the cells on the periphery and at the apical (inner) surface (Figure 4A). E-cadherin has a similar staining pattern, but with an increase in protein localization at either end of the primordial lumen (Figure 4A). Similar to calcium-treated Caco-2 monolayers,

PTPN14 localizes with E-cadherin and ZO-1 in mature wild-type cysts (Figure 4B). E-cadherin localizes to the basolateral surface and at lateral cell-cell contacts while ZO-1 lines the apical (luminal) surface. Collectively, these data indicate that PTPN14 localizes with AJ and TJ proteins during cell contact formation and during *ex vivo* lumen development.

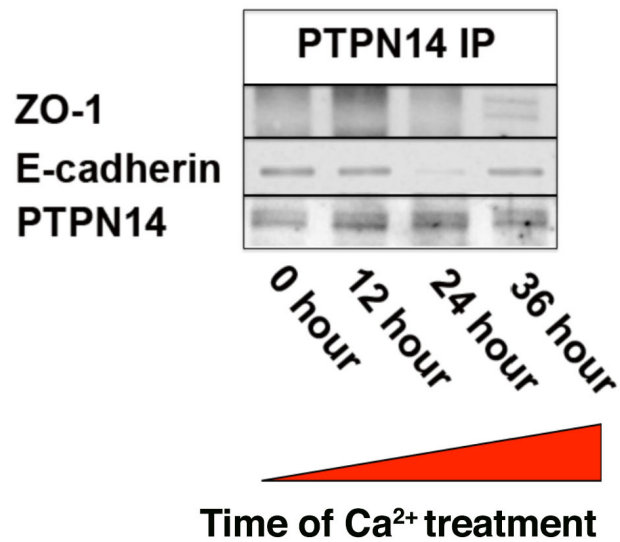
In line with previous observations, we found that PTPN14 localizes to cell:cell contacts (Figure 4C, lower panel). To determine if PTPN14 localizes with specific cell contacts during junctional development, we performed a calcium switch assay<sup>164-168</sup>. Cells placed in low-calcium media, which disrupts cadherin-mediated adhesion, (Figure 4C, top panel) show punctate co-localization of PTPN14, E-cadherin and the TJ protein ZO-1, indicative of spot (nascent) AJ. Following calcium depletion, addition of calcium triggers cell junction maturation and organization of PTPN14, E-cadherin and ZO-1 at cell:cell contacts (Figure 4C, bottom panel). Z-stack images show that PTPN14 overlaps with the AJ protein, E-cadherin, in both the early and late stages of junctional development (Figure 4D, 12-36 hours) and with the TJ protein, ZO-1, in the late stages of junctional development (Figure 4D, 24 hour and 36 hour). Immunoprecipitation of PTPN14-containing complexes during junction development found that PTPN14 physically associates with E-cadherin in the early and late stages of junctional development (0, 12, 36 hours) and ZO-1 during the late stages of junctional development (36 hours) (Figure 5).



**Figure 4. PTPN14 localizes to cell contacts during epithelial junction development.**

Localization of endogenous E-cadherin and PTPN14 in the primordial lumen of wild-type Caco-2<sup>BBE</sup> cells. E-cadherin and PTPN14 localize to the periphery and apical (inner) surface at the 2-cell stage with an increase in E-cadherin at the edge of the primordial lumen. White arrows point to E-cadherin clusters at the edges of the primordial lumen. Scale bars, 5μm. **B)** Localization of endogenous E-cadherin, PTPN14 and ZO-1 in mature wild-type 3D cysts. E-cadherin localizes basal surface and at cell:cell contacts while ZO-1 localizes to the apical (inner) surface of the cyst. PTPN14 overlaps with both E-cadherin and ZO-1. Scale bars, 50μm. **C)** Immunofluorescence of calcium starved (nascent junctions) and calcium treated cells (mature AJ and TJ) with antibodies against the TJ protein ZO-1, PTPN14 and E-cadherin. PTPN14 localizes to the cell membrane during both early and late junctional development. Scale bars, 20μm. **D)** Confocal z-stack images during junctional development (Ca<sup>2+</sup> treatment) showing that PTPN14 overlaps with E-cadherin in the early stages of junctional development (12 and 24 hour) and **E)** with both E-cadherin and ZO-1 in the late stages of junctional development (24 and 36 hour).





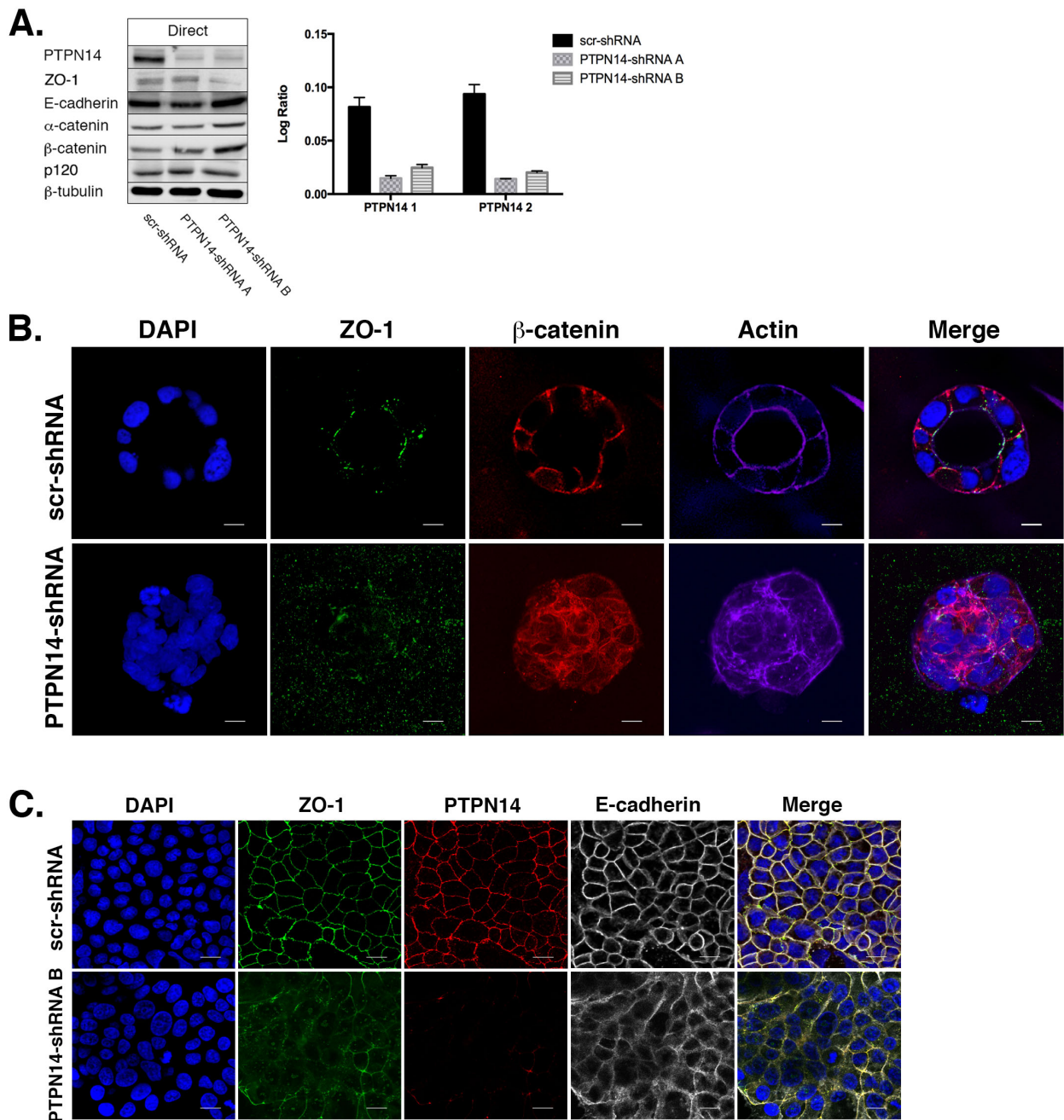
**Figure 5. PTPN14 associates with the AJ and TJ proteins during junction development.** Immunoprecipitation of PTPN14 containing protein complexes at different stages of junctional development as indicated by increasing time of calcium treatment. PTPN14 containing complexes were probed with antibodies against ZO-1, E-cadherin and PTPN14 to show that PTPN14 associates with ZO-1 and E-cadherin during junctional development.

### **PTPN14 is necessary for lumen formation and junction organization**

Development of epithelial cell junctions is directly tied to formation of apicobasal polarity and lumen formation<sup>84,94</sup>. The observation that PTPN14 localizes with both AJ and TJ proteins during junctional development led us to question whether PTPN14 regulates junction organization. To this end, we infected Caco-2 cells with a scrambled shRNA and two independent shRNA constructs targeting PTPN14 (Figure 6A). qRT-PCR analysis and direct Western blot show a marked decrease in PTPN14 RNA and protein with no change in AJ protein levels (Figure 6A). To determine if PTPN14 has a role in lumen development, we performed a 3D culture lumen formation assay on scramble control and PTPN14-shRNA cells. In control cells ZO-1 marks TJ at the apical surface, actin concentrates at the apical domain and at cell contacts, and  $\beta$ -catenin, a known PTPN14 substrate<sup>67</sup> and a component of the AJ, is distributed to the basolateral portion of the lumen (Figure 6B, top panel). Surprisingly, PTPN14-shRNA cells grown in 3D failed to form lumens, lacked ZO-1 and  $\beta$ -catenin organization and had a disorganized actin cytoskeleton (Figure 6B) indicating that PTPN14 is necessary for lumen formation and organization of cell contacts during epithelial morphogenesis. In 2D culture, PTPN14-shRNA cells showed diminished levels of PTPN14, lacked organization of ZO-1 and had reduced localization of E-cadherin at the membrane compared to control cells (Figure 6C) suggesting a role for PTPN14 during cell junction development.

### **PTPN14 depletion disrupts formation of functional epithelial cell junctions**

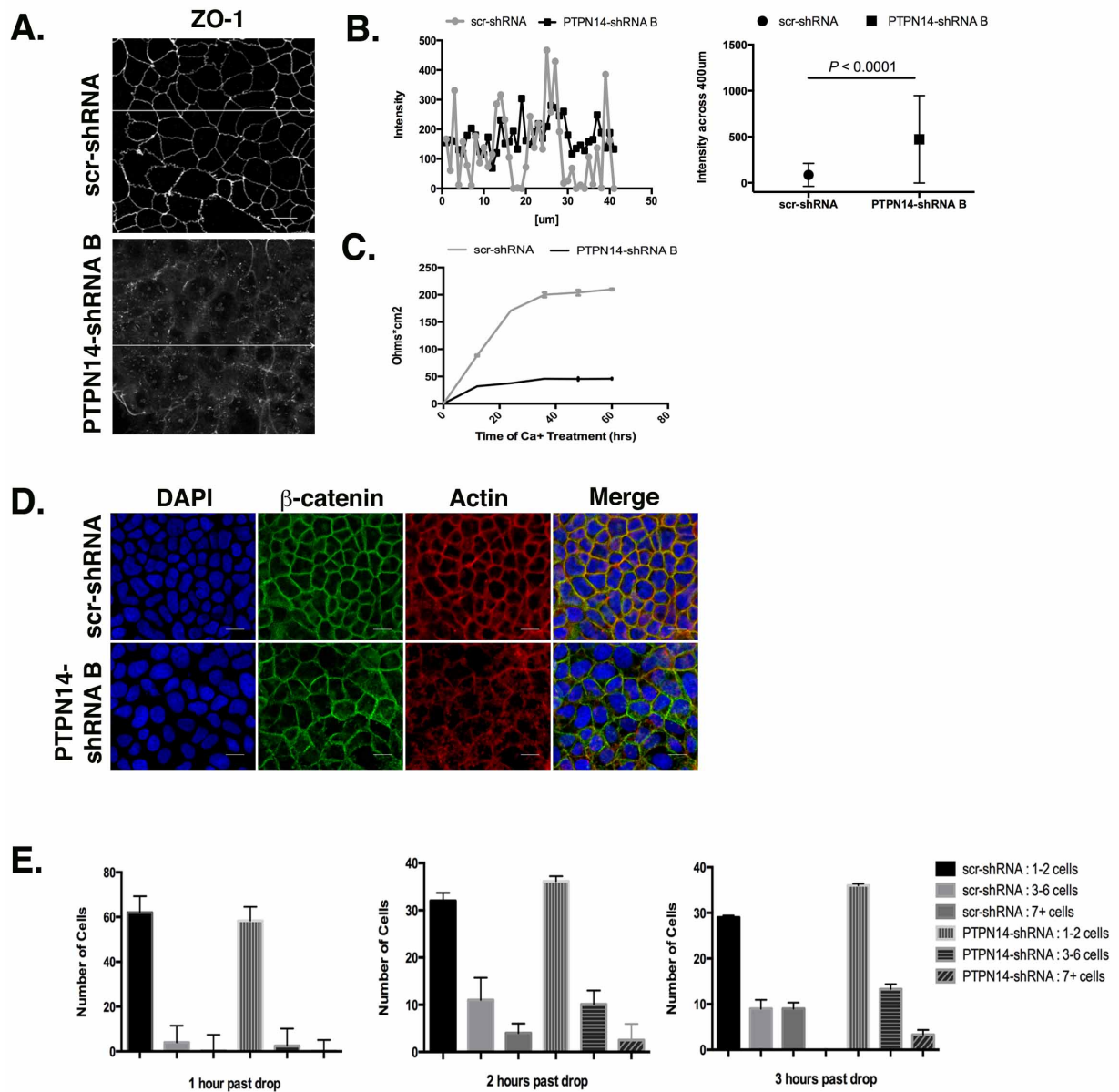
TJs regulate cell polarity by serving as a fence that restricts apical and basolateral membrane proteins to their respective cellular compartments<sup>24-26</sup>. The observation that PTPN14-shRNA cells do not form polarized epithelial cysts suggests that these cells have altered cell



**Figure 6. PTPN14 is necessary for lumen formation and junction organization** **A)** Direct Western analysis and quantitative qRT-PCR using two independent probes demonstrates knockdown of PTPN14 in two separate shRNA-mediated knockdown constructs of PTPN14 (PTPN14-shRNA) in *Caco-2*<sup>BBE</sup> cells but no alteration of overall TJ or AJ protein levels. **B)** PTPN14-shRNA cells exhibit a no lumen phenotype, with mislocalization of ZO-1 and E-cadherin, in a 3D cyst formation culture assay as compared to control (scr-shRNA) cells. Scale bars, 20μm. **C)** Immunofluorescence of scramble (scr-shRNA) and PTPN14-shRNA expressing cells using antibodies against ZO-1, PTPN14 and E-cadherin demonstrating decreased PTPN14 alters TJ and AJ protein localization at cell:cell contacts. Scale bars, 20μm.

contacts. To determine if PTPN14-shRNA cells form functional TJs we first quantitated the distribution of ZO-1 in PTPN14-shRNA cells compared to control cells. Control cells have ZO-1 staining at distinct cell contacts compared to the diffuse and mislocalized membrane staining observed in PTPN14-shRNA cells (Figure 7A). Intensity profile analysis revealed a lack of defined peak in intensity at cell-cell contacts in PTPN14-shRNA cells (Figure 7B). We next measured trans-epithelial resistance (TER) over time following a calcium switch (as detailed in Figure 4C)<sup>84</sup> to determine if the altered ZO-1 cell junction localization in PTPN14-shRNA cells corresponded with the loss of TJ function. Control cells showed an increase in TER with increased time of calcium treatment, whereas cells expressing decreased levels of PTPN14 displayed disrupted TER formation (Figure 7C). This finding indicates that PTPN14 activity is crucial for epithelial TJ function.

We previously demonstrated that formation of functional TJs in epithelial cells is dependent upon both AJ proteins and cell polarity proteins<sup>84</sup>. To determine if decreased levels of PTPN14 altered AJ formation we first examined the localization of  $\beta$ -catenin, an AJ protein and PTPN14 substrate<sup>67</sup> in control and PTPN14-shRNA Caco cells. This analysis revealed a diffuse staining pattern at the membrane, similar to what we observed in 3D cyst cultures (compare Figure 6B & Fig. 7D). As we also observed altered actin organization in PTPN14-shRNA cells in 3D cyst cultures, we next sought to determine if actin organization was altered in monolayer cultures. We stained control and PTPN14 depleted cells with Phalloidin to label the actin cytoskeleton. PTPN14-shRNA cells displayed evidence of disrupted actin organization at cell contacts (Figure 7D), suggesting that the actin cytoskeleton is unable to sense and respond to cell contacts in these cells. To determine if the altered actin organization observed in PTPN14-shRNA cells resulted from alterations in AJ function, we assessed the capacity of cells to form



**Figure 7. PTPN14 depletion disrupts formation of functional epithelial cell junctions.**

**A)** Intensity profile staining of ZO-1 in scr- and PTPN14-shRNA expressing *Caco-2*<sup>BBE</sup> cells. Scale bars, 20µm. **B)** Quantitation of ZO-1 staining intensity plotted across the cell membrane. Scr-shRNA cells display peaks of ZO-1 intensity while ZO-1 is mislocalized and displays a diffuse staining pattern in PTPN14-shRNA cells. **C)** Trans-epithelial resistance (TER) of confluent scr-shRNA or PTPN14-shRNA expressing *Caco-2*<sup>BBE</sup> cells revealed decreased TJ function in cells depleted of PTPN14. Values equal mean (n=2) +/- SD. **D)** Phalloidin staining revealed altered actin organization in PTPN14 knockdown cells.  $\beta$ -catenin localizes to the membrane in PTPN14-shRNA cells. Scale bars, 20µm. **E)** PTPN14-shRNA cells fail to form large cell clumps, indicative of stabilized AJ, in hanging drop assays.

functional AJs in a hanging drop assay<sup>84,130</sup>. Control cells showed a step-wise increase in 3-6 cell and 7+ cell clumps with increasing time demonstrating their ability to initiate and stabilize cadherin-mediated adhesion (Figure 7E). Conversely, PTPN14-shRNA cells were able to form 3-6 cell clumps like control cells, but were unable to form larger clumps suggesting that although these cells are able to initiate cadherin contacts, they are unable to stabilize AJ contacts. The lack of stabilized AJs, together with the alterations in actin organization at cell contacts, indicates that PTPN14 function is necessary for AJ stabilization during epithelial morphogenesis.

### **PTPN14 regulates $\beta$ -catenin-Y654 phosphorylation**

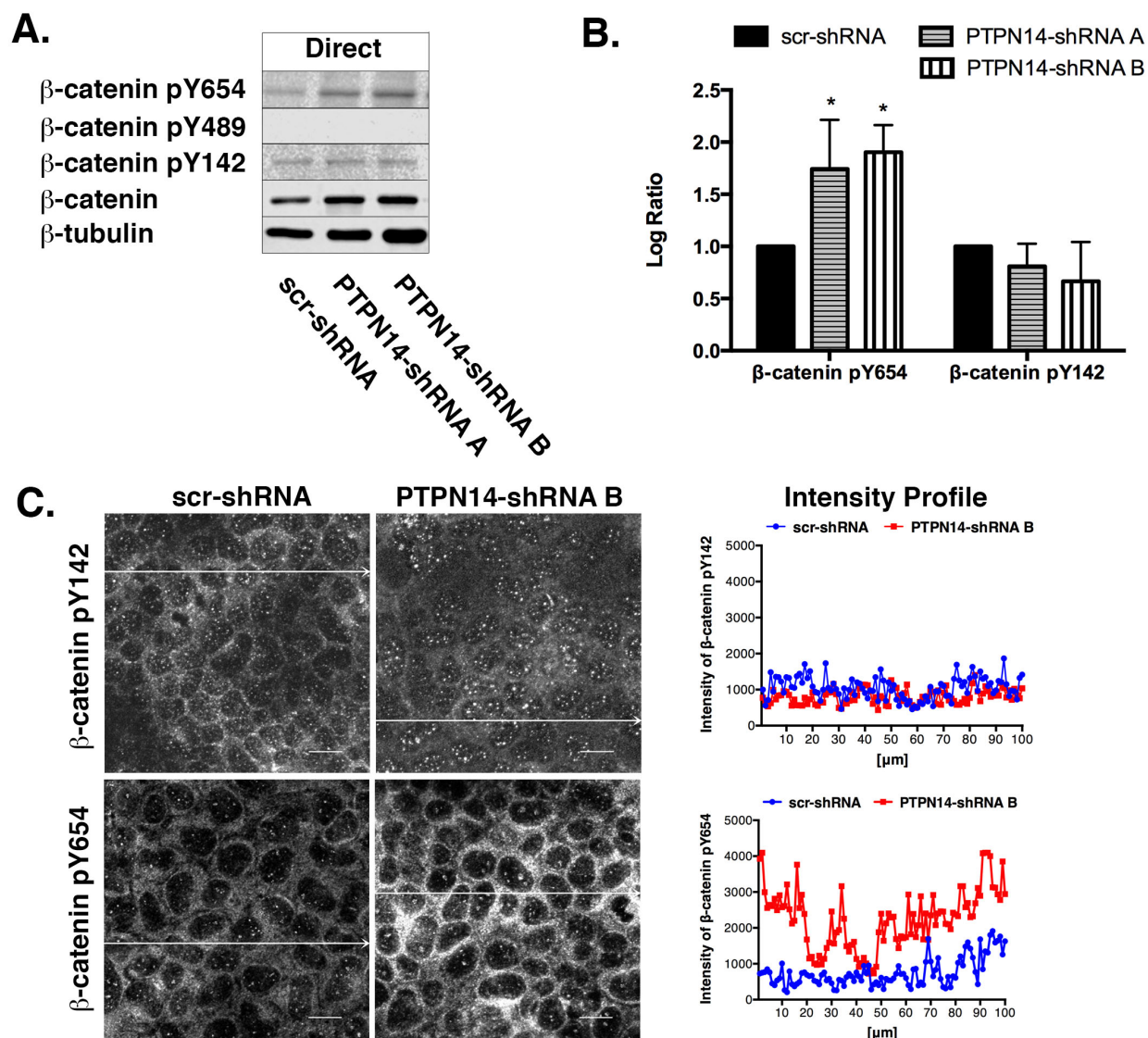
Previous reports identified  $\beta$ -catenin as a substrate of PTPN14 in polarized kidney epithelial cells<sup>67</sup>. However, the tyrosine residue at which PTPN14 dephosphorylates  $\beta$ -catenin remained unknown<sup>67</sup>.  $\beta$ -catenin contains at least three potential sites for PTPN14-mediated dephosphorylation: tyrosine residues 142, 489 and 654. Phosphorylation at these tyrosine residues controls the interaction of  $\beta$ -catenin with other AJ proteins, thereby aiding in regulation of cell adhesion. Phosphorylation of  $\beta$ -catenin-Y142 by the FER and Fyn kinases reduces the binding of  $\beta$ -catenin with the AJ protein,  $\alpha$ -catenin, and facilitates the interaction of  $\beta$ -catenin with the human proto-oncogene product, BCL-9<sup>169-172</sup>. *In vitro* kinase assays demonstrated that  $\beta$ -catenin-Y489 is phosphorylated by Abl kinase, eliciting dissociation of  $\beta$ -catenin from N-cadherin and localization of phospho- $\beta$ -catenin-Y489 to the nucleus where it is able to promote transcriptional activation<sup>173</sup>. Additionally, pp60<sup>c-src</sup> kinase phosphorylates  $\beta$ -catenin at Y654, which regulates AJ-mediated adhesion<sup>174</sup>. Our observations that depletion of PTPN14 disrupts AJ formation led us to assess these candidate tyrosine residues of  $\beta$ -catenin to determine if PTPN14 regulates a specific tyrosine phosphorylation site. Confluent monolayers of control shRNA and PTPN14-shRNA Caco-2 cells were collected and probed with phospho-specific antibodies towards  $\beta$ -

catenin-Y142, -Y489 or -Y654. Phospho- $\beta$ -catenin-Y489 was not detected in either control or PTPN14 depleted cells (Figure 8A); this finding is not unexpected given the lack of N-cadherin expression in these cells (data not shown). Additionally, we observed no change in phospho- $\beta$ -catenin-Y142 levels between the two cell lines (Figure 8A). An increase in phospho- $\beta$ -catenin-Y654 was observed in PTPN14-shRNA cells compared to scr-shRNA cells (Figure 8A) suggesting PTPN14 can dephosphorylate  $\beta$ -catenin at Y654. Densitometry analysis of four independent confluent cell lysates showed a near two-fold increase in tyrosine phosphorylation at  $\beta$ -catenin-Y654 in PTPN14-shRNA cells (Figure 8B). Similarly, indirect immunofluorescence staining of confluent cells revealed increased levels of phospho- $\beta$ -catenin-Y654 staining at sites of cell contact in PTPN14-depleted cells, but no change in levels of pY142 (Figure 8C). Intensity profiles across an approximate 400 $\mu$ m cell layer (white line in Figure 8C) of both cell types indicated the intensity of phospho- $\beta$ -catenin-Y142 staining was not increased in PTPN14 depleted cells compared to control cells similar to our observation by direct Western analysis (Figure 8A). Intensity profiles for phospho- $\beta$ -catenin-Y654 in both cell types showed an increase in staining intensity across the monolayer of PTPN14-shRNA cells (Figure 8C) suggesting PTPN14 regulates  $\beta$ -catenin-Y654 phosphorylation near sites of cell contact.

### **PTPN14 regulates membrane mobility of E-cadherin at cell contacts**

Src-mediated  $\beta$ -catenin-Y654 phosphorylation disrupts E-cadherin-dependent cell adhesion<sup>161,174</sup>. We observed that cells depleted of PTPN14 have altered AJ function and increased  $\beta$ -catenin-Y654 phosphorylation. In addition, previous studies demonstrate that



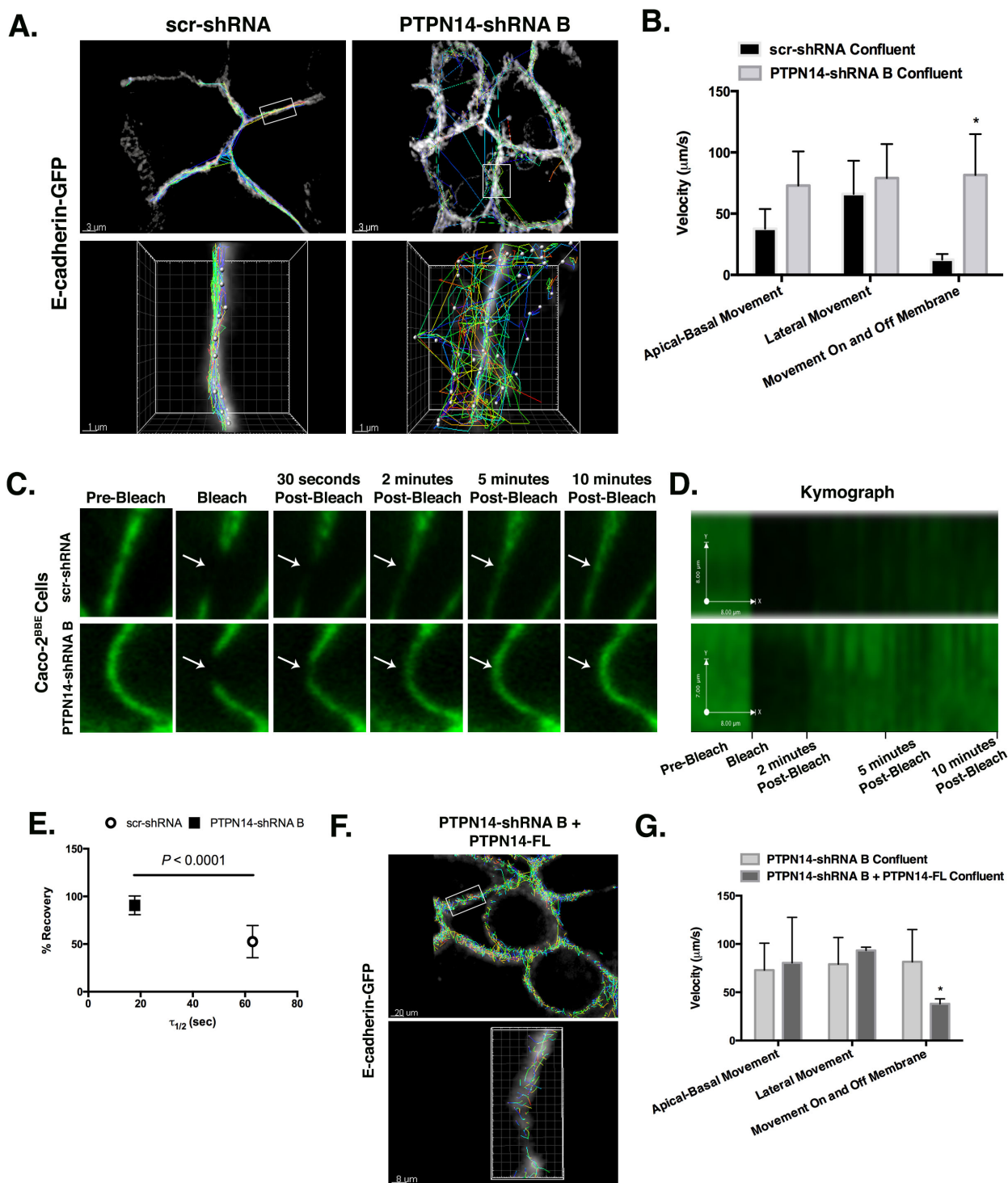


**Figure 8. PTPN14 regulates β-catenin-Y654 phosphorylation.** **A)** Direct Western analysis of indicated Caco-2<sup>BBE</sup> cells showing an increase of β-catenin Y654 phosphorylation in two separate PTPN14-shRNA expressing cells as compared to scramble-shRNA control cells. PTPN14 knockdown did not alter levels of β-catenin Y142 and Y489 phosphorylation. **B)** Densitometry comparison of β-catenin pY654 and pY142 between scramble control (scr-shRNA) and two PTPN14-shRNA expressing cells showing a significant increase in β-catenin Y654 phosphorylation in cells that have decreased PTPN14. Values equal mean (n=4) +/- SD (\*=p < 0.05). Asterisks denote a significant difference between the experimental and control values, as assessed using the T-test p < 0.05. **C)** Immunofluorescence quantification of phospho-β-catenin-Y142 (top panels) or phospho-β-catenin-Y654 (bottom panels) by using the brightness intensities of each pixel. PTPN14 knockdown cells display an increase in phospho-β-catenin-Y654 (red line, bottom panel) as plotted on the graph in the right panel. Scale bars, 20μm.

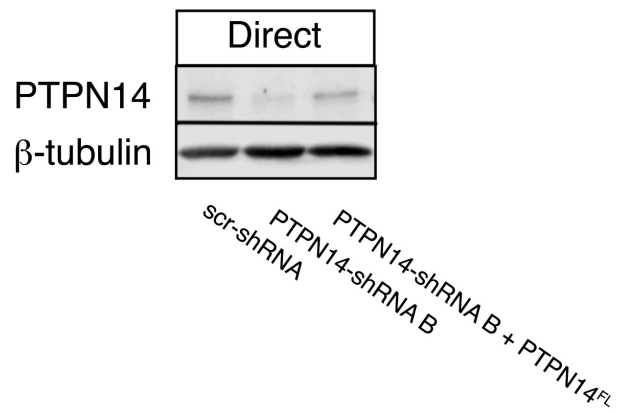


E-cadherin and  $\beta$ -catenin membrane mobility have a role in regulating cadherin mediated adhesion<sup>175</sup>. This led us to examine the membrane mobility of E-cadherin in PTPN14-shRNA Caco-2 cells. In order to address characteristics of E-cadherin mobility, we performed live-cell imaging studies to look at E-cadherin at cell:cell contacts. E-cadherin was visualized in Caco-2 cells by stable infection with lentivirus expressing GFP-tagged E-cadherin while simultaneously expressing shRNA targeting endogenous E-cadherin to eliminate any effects of protein overexpression<sup>176</sup>.

In scramble control Caco-2 cells GFP-E-cadherin primarily moves laterally along cell contacts with some apicobasal movement in the cell contacts as previously described<sup>176</sup> (Figure 9A and B). By contrast, PTPN14-shRNA cells display a large increase in GFP-E-cadherin movement on and off the membrane at cell contacts (Figure 9A and B). Although the velocity of lateral (y plane) GFP-E-cadherin movement was similar between PTPN14-shRNA and control cells (Figure 9B), we observed significant increases in apicobasal (z plane) and movement on and off the membrane (x plane) in cells with decreased PTPN14 (Figure 9B). These results support a role for PTPN14 in regulating E-cadherin mobility at cell contacts. Next, we used Fluorescence Recovery After Photobleaching (FRAP) to examine the dynamics and turnover of E-cadherin at the cell membrane. In control cells, GFP-E-cadherin half-life recovery after photobleaching averaged 60 seconds with the highest percentage of recovery at 68%. Conversely, cells with decreased PTPN14 had an average E-cadherin-GFP half-life of 17 seconds and the highest percentage of recovery at 100% (Figure 9C-E). We expressed a full-length shRNA-resistant PTPN14 construct in PTPN14-shRNA cells (Figure 10) and examined E-cadherin mobility by live-cell imaging. We observed a decrease in apicobasal movement and movement on and off the membrane with no change in lateral movement (Figure 9F). Quantitative analysis



**Figure 9. PTPN14 regulates membrane mobility of E-cadherin at cell contacts.** **A)** Live-cell imaging of GFP-E-cadherin in scr- and PTPN14-shRNA Caco-2<sup>BBE</sup> cells shows an overall increased scattered movement of E-cadherin in PTPN14-depleted cells (top panel). Single cell:cell contact analysis confirms increased movement on and off the membrane in cells with decreased PTPN14 (bottom panel). **B)** Quantitative analysis of E-cadherin movement in confluent scr- and PTPN14-shRNA cells shows a change in movement on and off the cell membrane (x plane). Values equal mean +/- SD (\*=p <0.05). **C)** FRAP analysis of scr- (top) and PTPN14-shRNA (bottom) cells shows an increase in E-cadherin-GFP recovery following photo-bleaching in PTPN14-shRNA cells compared to control (scr-shRNA) cells indicating an increase in E-cadherin movement at cell:cell contacts. Arrows demarcate photo-bleached spots. **D)** Kymograph chart of FRAP data representing the region of interest (ROI) shown in (C). **E)** Plotted parameters for recovery kinetics with an average of 20 ROIs for each cell type demonstrating a significant increase in recovery time in PTPN14-depleted cells. **F)** Live-cell imaging of E-cadherin-GFP in PTPN14-shRNA cells expressing sh-RNA resistant full-length PTPN14. Increased movement of E-cadherin-GFP observed in PTPN14-shRNA cells is blocked by expression of PTPN14 (top panel). Single cell:cell contact analysis in full-length re-expression cells demonstrates decreased E-cadherin mobility in PTPN14-shRNA cells re-expressing PTPN14 (bottom panel). **G)** Quantitative comparison of E-cadherin movement between cells with depleted PTPN14 (PTPN14-shRNA B, light bars) and re-expression of full-length PTPN14 (dark bars) demonstrate that re-expression of PTPN14 reduces E-cadherin movement on and off the membrane. Values equal mean +/- SD (\*=p <0.05).



**Figure 10. Re-expression of full-length PTPN14.** Direct Western blot analysis confirming endogenous levels of PTPN14 in control cells, decreased PTPN14 through shRNA interference and re-expression of PTPN14 using a construct expressing shRNA resistant full length PTPN14.

determined that re-expression of full-length PTPN14 reduces the increased movement of E-cadherin on and off the membrane observed in cells with decreased PTPN14 (Figure 9G) indicating that PTPN14 functions to confine E-cadherin mobility at cell contacts.

**PTPN14 stabilizes E-cadherin mobility by opposing Src kinase activity at  $\beta$ -catenin-Y654.**

Our observations that Caco-2 cells depleted of PTPN14 have increased phospho-  $\beta$ -catenin-Y654 and increased E-cadherin movement on and off the membrane led us to ask if PTPN14 phosphatase activity was necessary for regulating E-cadherin membrane movement. To directly determine if regulation of E-cadherin mobility at cell contacts is dependent upon PTPN14 phosphatase activity, we performed live-cell imaging experiments in PTPN14 shRNA Caco-2 cells expressing shRNA-resistant phosphatase dead PTPN14 mutant (PTPN14<sup>C1121S</sup>)<sup>71</sup> and GFP-E-cadherin. Expression of PTPN14<sup>C1121S</sup> had no effect on E-cadherin movement on and off the membrane (Figure 11A, left panel). Also, the velocity of E-cadherin movement on and off the membrane does not change between decreased PTPN14 (PTPN14-shRNA B, left panel) and over expression of PTPN14<sup>C1121S</sup> in Caco-2 cells with decreased PTPN14 (Figure 11B, middle panel). Through this, we determined that the control of E-cadherin mobility at the membrane is a phosphatase-dependent role of PTPN14.

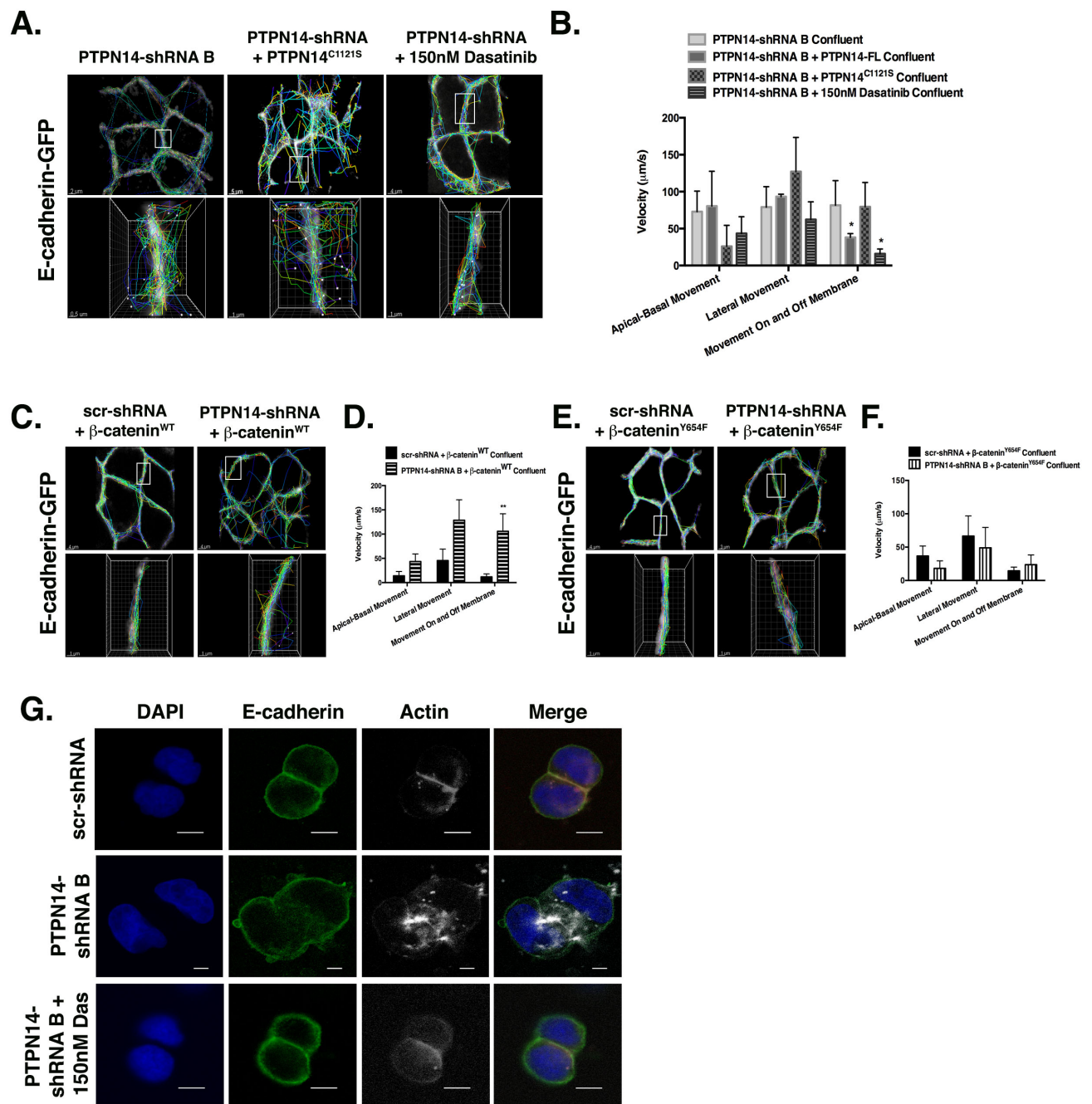
From our observations that PTPN14 phosphatase activity is required to regulate E-cadherin mobility, we then investigated if PTPN14 opposed Src kinase activity, which is known to phosphorylate  $\beta$ -catenin-Y654 at cell contacts<sup>161</sup>. PTPN14-shRNA Caco-2 cells were pre-treated with 150nM of Dasatinib, a small molecule inhibitor of the Src family kinases (SFK)<sup>177-179</sup> and GFP-E-cadherin movement was observed by live-cell imaging (Figure 12). Dasatinib treatment blocked increased movement of E-cadherin on and off the membrane in cells with decreased PTPN14 (Figure 11A, right panel) and quantitative evaluation of E-cadherin mobility

revealed E-cadherin movement on and off the membrane was decreased in a similar fashion to re-expression of full-length PTPN14 (Figure 11B, right panel). These data indicate that PTPN14 phosphatase activity opposes Src kinase activity at cell contacts.

We next asked if the increased movement of E-cadherin observed in cells with decreased levels of PTPN14 could be rescued by preventing phosphorylation of  $\beta$ -catenin-Y654. Whereas over-expression of  $\beta$ -catenin<sup>WT</sup> did not alter the increased movement of E-cadherin at the membrane in cells with depleted levels of PTPN14 (Figure 11C), cells expressing the non-phosphorylatable  $\beta$ -catenin<sup>Y654F</sup> mutant showed a significant decrease in overall E-cadherin movement (Figure 11E), reaching levels similar to those in control cells (Figure 11F). These results indicate that PTPN14-mediated dephosphorylation of  $\beta$ -catenin-Y654 stabilizes E-cadherin mobility at cell contacts.

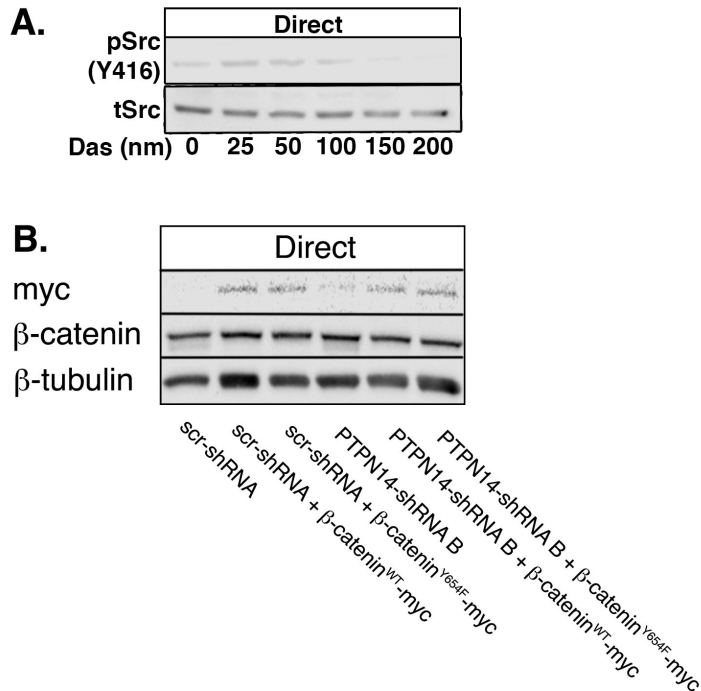
### **PTPN14 activity is necessary for E-cadherin-mediated lumen formation**

Caco-2 lumen formation derives from a single cell that polarizes centrosomes under an actin-rich cap prior to the first cell division. Although the role of E-cadherin-mediated adhesion during early lumen formation is not understood<sup>94,180</sup>, our results (Figure 4A and B) suggest that E-cadherin localization is highly regulated at this stage of lumen morphogenesis. To determine how PTPN14-mediated regulation of E-cadherin affects early lumen formation, we first examined E-cadherin localization immediately following the first cell division in Caco-2 3D cultures. In control cells E-cadherin localized around the outside of both cells and to an E-cadherin bridge that connects the newly divided cells (Figure 11G, top panel). Actin concentrates at this E-cadherin bridge. In PTPN14-shRNA cells, the E-cadherin bridge fails to form (80%) and actin does not organize to the cell:cell contact which will serve as the site for initial lumen formation (Figure 11G, middle panel). Inhibition of Src kinase activity rescues formation of the

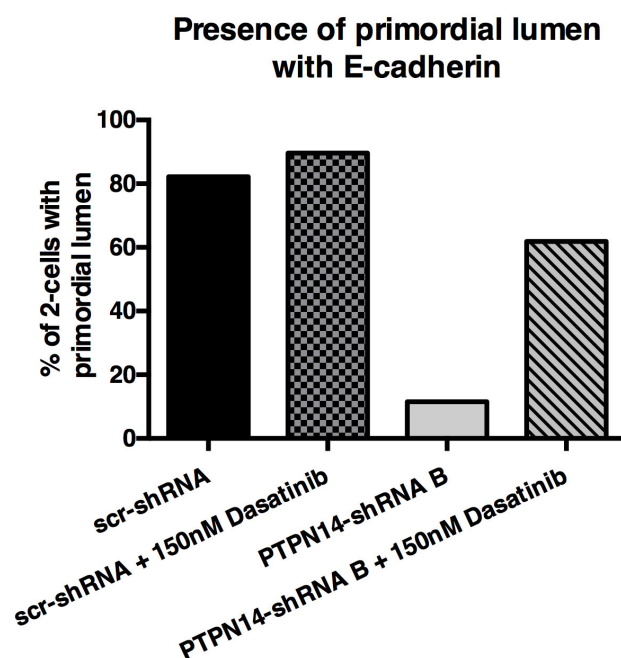


**Figure 11. PTPN14 stabilizes E-cadherin mobility by opposing Src kinase activity at  $\beta$ -catenin-Y654.** **A)** Live-cell imaging of E-cadherin-GFP in PTPN14-shRNA Caco-2<sup>BBE</sup> cells expressing a phosphatase -dead mutant, PTPN14<sup>C1121S</sup>, and cells treated with 150nM of the Src inhibitor, Dasatinib. PTPN14<sup>C1121S</sup> does not alter the increased mobility of E-cadherin observed in PTPN14-depleted cells; by contrast, treatment with Dasatinib decreases mobility of E-cadherin at the membrane in PTPN14-shRNA cells (top panel). Bottom panel represents a single cell:cell contact. **B)** Quantification of E-cadherin movement at cell:cell contacts in full-length re-expression and Dasatinib treatment, but not phosphatase dead PTPN14, significantly decreases E-cadherin movement on and off the membrane of cells depleted of PTPN14. Values equal mean  $\pm$  SD (\*= $p < 0.05$ ). **C)** Live-cell imaging of E-cadherin-GFP in scr- and PTPN14-shRNA cells overexpressing wild-type  $\beta$ -catenin (scr-shRNA +  $\beta$ -catenin<sup>WT</sup> and PTPN14-shRNA +  $\beta$ -catenin<sup>WT</sup>, respectively). Expression of wild-type  $\beta$ -catenin<sup>WT</sup> does not alter the increased mobility of E-cadherin observed in PTPN14-shRNA cells (top panel). Bottom panel is a single cell:cell contact view of each cell type. **D)** Quantitative analysis of E-cadherin movement in cells from (C). Values equal mean  $\pm$  SD (\*\*= $p < 0.005$ ). **E)** Live-cell imaging of E-cadherin-GFP in scr- and PTPN14-shRNA cells overexpressing non-phosphorylatable mutant of  $\beta$ -catenin at tyrosine 654 ( $\beta$ -catenin<sup>Y654F</sup>). Expression of  $\beta$ -catenin<sup>Y654F</sup> in PTPN14-shRNA cells decreases overall E-cadherin movement (top panel). Single cell:cell contact analysis also reveals a decrease in E-cadherin movement at the cell membrane (bottom panel). **F)** Quantitation of E-cadherin movement in cells from (E). Expression of  $\beta$ -catenin<sup>Y654F</sup> rescues the increased movement of E-cadherin in cells with decreased PTPN14. **G)** Immunofluorescence staining of Caco-2<sup>BBE</sup> 3D cultures with an antibody against E-cadherin and Phalloidin to label actin at the 2-cell stage. Cells with decreased PTPN14 expression exhibit disrupted actin and E-cadherin localization at the 2-cell stage. Treatment with 150nM Dasatinib rescues the localization of E-cadherin and actin demonstrating that Src inhibition can rescue the effects of decreased PTPN14 during early lumen formation. Bars, 10 $\mu$ m for top and bottom panels; 5 $\mu$ m for middle panel.





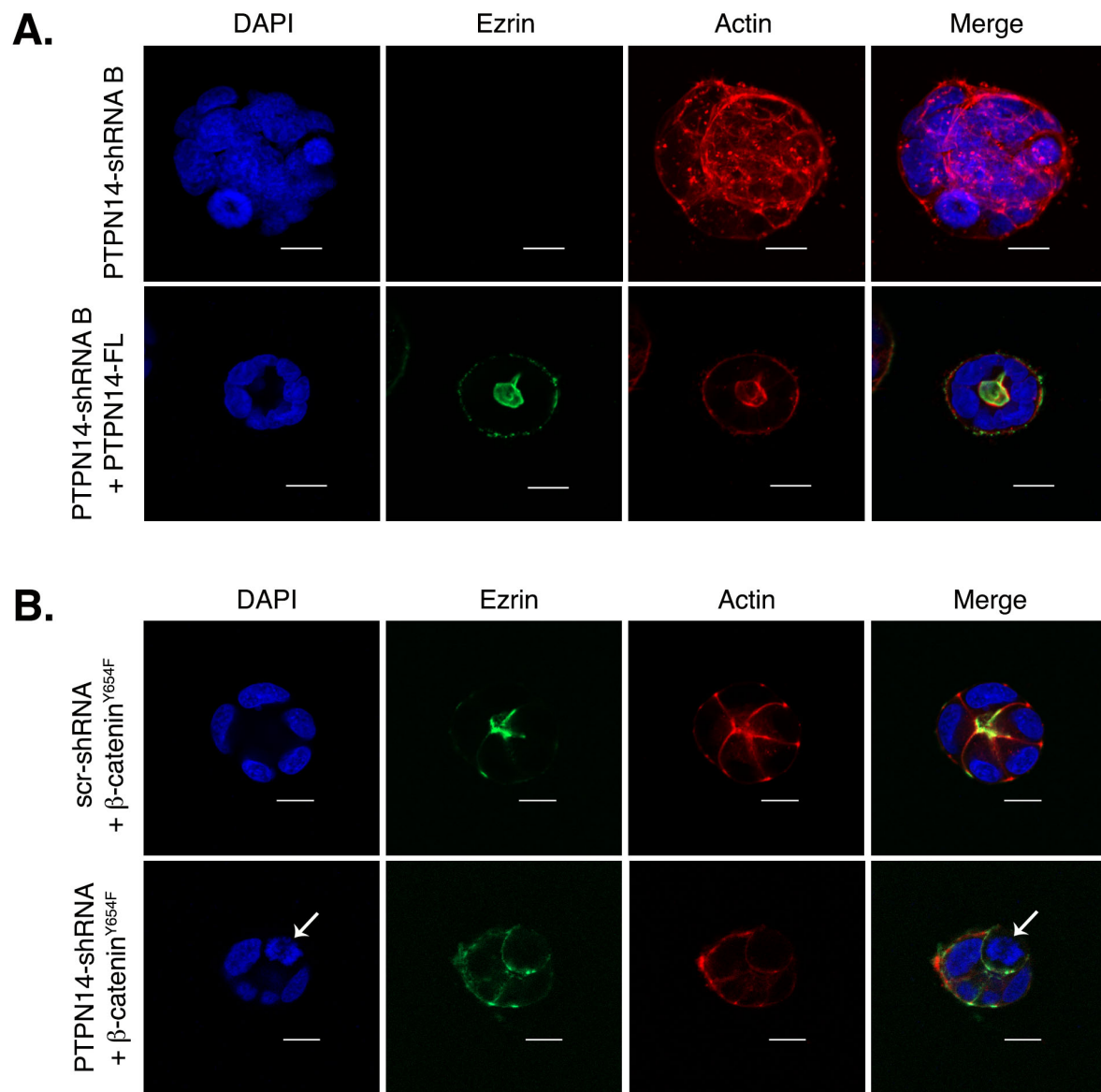
**Figure 12.  $\beta$ -catenin protein expression and measurement of Src activity.** Confluent Caco-2 cells were treated with the indicated concentrations of Dasatinib two times per day for two days and collected for direct Western. Lysates were probed with antibodies detecting total Src (tSrc) or phosphorylated Src (Y416) to show that 150 and 200 nM concentrations of Dasatinib decreased Src activity in Caco cells. **B)** scr-shRNA or PTPN14-shRNA Caco cells were infected with either Myc tagged wild-type  $\beta$ -catenin ( $\beta$ -catenin<sup>WT</sup>-myc) or a non-phosphorylatable  $\beta$ -catenin-Y654F mutant ( $\beta$ -catenin<sup>Y654F</sup>-myc). Direct Westerns of the indicated lysates were probed with antibodies recognizing the Myc tag to show expression of the indicated constructs or total  $\beta$ -catenin to show that overall  $\beta$ -catenin levels do not change in cells expressing the indicated constructs.



**Figure 13. Quantitation of E-cadherin bridge during early lumen formation.** The presence of an E-cadherin bridge in early 2-cell cysts was counted in scr-shRNA, scr-shRNA + 150nM Dasatinib, PTPN14-shRNA and PTPN14-shRNA + 150nM Dasatinib. Primordial lumen formation is decreased by 80% in cells with decreased PTPN14 and can be partially rescued by treatment with 150nM of Dasatinib.

E-cadherin bridge and actin localization at the initial cell contact (Figure 11G, bottom panel; Figure 13). These data demonstrate that PTPN14 opposes Src kinase activity on  $\beta$ -catenin at the two-cell stage of lumen formation to establish an E-cadherin bridge in the early stages of lumen formation.

To understand if control of E-cadherin mobility by PTPN14 has a functional role after the two-cell stage, we re-expressed shRNA-resistant PTPN14 in PTPN14-shRNA cells and examined 3D lumen formation. In agreement with our previous observations, (Figure 6B, bottom panel), cells depleted of PTPN14 do not form a lumen in 3D. We also observed mislocalization of ZO-1 and  $\beta$ -catenin, as well as actin disorganization. Interestingly, we also noted a complete loss of the apical protein, Ezrin, in 3D cysts that lack PTPN14 (Figure 14A, top panel). When full-length PTPN14 is expressed in PTPN14-shRNA cells, a lumen forms in Caco-2 cells (Figure 14A). Ezrin concentrates to the apical domain and actin is concentrated at the surface of the lumen (Figure 14A, bottom panel). This indicates that PTPN14 re-expression is sufficient to promote the formation of a single lumen in Caco-2 cells. To determine if PTPN14 establishes this lumen by regulation of phospho- $\beta$ -catenin-Y654, we expressed  $\beta$ -catenin<sup>Y654F</sup> in either control shRNA cells or cells with decreased PTPN14 and witnessed a similar outcome as what is observed when we express full-length PTPN14. These cells localize Ezrin to the apical domain and actin is organized around the primordial lumen (Figure 14B, bottom panel). We also observed polarized cell division occurring parallel to the apical surface, which is indicative of establishment of apicobasal polarity in the cyst (Figure 14B, bottom panel, white arrow). These data establish a functional role for PTPN14 in 3D cyst development in intestinal epithelial cells through the regulation of  $\beta$ -catenin-Y654.



**Figure 14. PTPN14 activity is necessary for E-cadherin-mediated lumen formation**

**A)** PTPN14-shRNA cells have a no lumen phenotype and lose the apical marker, Ezrin, in mature 3D cysts and exhibit actin disorganization (top panel). Expression of shRNA-resistant full-length PTPN14 rescues actin and Ezrin localization in 3D cysts (bottom panel) indicating that PTPN14 is required for apical-basal polarity in Caco-2<sup>BBE</sup> cells. Scale bars, 20 $\mu$ m. **B)** Overexpression of a non-phosphorylatable mutant of  $\beta$ -catenin at Y654 ( $\beta$ -catenin<sup>Y654F</sup>) in PTPN14 sh-RNA cells promotes Ezrin localization and actin organization towards the early lumen (bottom panel). Expression of  $\beta$ -catenin<sup>Y654F</sup> also promotes polarized cell division around the early lumen in PTPN14 sh-RNA cells; white arrow demarcates a cell that is correctly dividing parallel to the lumen. Scale bars, 20 $\mu$ m.

## Discussion

We present data indicating that the non-receptor tyrosine phosphatase PTPN14 opposes Src-mediated disruption of cadherin-mediated cell adhesion thereby resulting in stabilization of E-cadherin at cell contacts. PTPN14-mediated stabilization of E-cadherin at cell contacts promotes functional AJ adhesion, TJ adhesion and polarized organization of three dimensional cell structures. We show that endogenous PTPN14 localizes to cell contacts in both 2D and 3D cultures of Caco-2<sup>BBE</sup> epithelial cells, co-localizing with AJ and TJ proteins during specific stages of cell junction development. Moreover, we demonstrate that PTPN14 phosphatase activity is necessary for formation of functional AJ, TJ and polarized lumens, and that this activity regulates Src-mediated phosphorylation of  $\beta$ -catenin-Y654 thereby stabilizing E-cadherin at cell contacts. Coupled with our previous work that demonstrated a requirement for AJ proteins to establish apicobasal polarity and functional TJ in epithelial cells<sup>84</sup>, our new data support the idea that establishment of stable AJs is critical for junction development and organization of epithelial cells within the tissue to maintain homeostasis. Although these data indicate the importance of the AJ during tissue development, we lack a complete understanding of how epithelial cells with established apicobasal polarity and cell junctions respond to altered function of PTPN14 (or that of other junctional proteins). Observations reporting mutations or changes of expression of PTPN14 in various cancers would suggest that regulation of PTPN14 levels may be critical for maintenance of epithelial tissues. It does appear, however, that small changes in PTPN14 activity can greatly alter epithelial tissues<sup>65</sup>. The identification of protein(s) that regulate PTPN14 should provide insight into the capacity of PTPN14 activity to alter cell adhesion and promote or accelerate disease progression.

Regulation of cadherin-mediated adhesion by growth factors can occur through numerous signaling pathways and target different proteins within the AJ. Phosphorylation of  $\beta$ -catenin by

Src regulates E-cadherin adhesion, but it does so in the context of an intact E-cadherin-  $\beta$ -catenin protein complex<sup>144,172,181</sup>. Constitutive, but low-level Src-dependent  $\beta$ -catenin-Y654 phosphorylation enables the continual lateral movement of E-cadherin at cell contacts; increased growth factor-dependent signaling through Src diminishes adhesion<sup>159</sup> and allows cells to divide and repopulate the tissue. How PTPN14 activity responds to this varying level of Src activity and whether there is a built in feedback loop remains to be seen. Additionally, how PTPN14-mediated regulation of  $\beta$ -catenin phosphorylation cooperates with other AJ phosphorylation events that alter adhesion, including the phosphorylation of E-cadherin<sup>182,183</sup> and p120-catenin<sup>172,174,184,185</sup>, will be a critical area to examine.

Cadherin-mediated adhesion promotes apicobasal polarity and organization of epithelial tissues, including tissues containing lumens. The inability of intestinal cells depleted of PTPN14 to form a lumen is reminiscent of the no-lumen phenotype observed in Caco cells expressing Ezrin <sup>$\Delta$ act</sup>, a dominant negative form of the apical protein Ezrin<sup>94</sup>. In both *C. elegans*<sup>186,187</sup> and mouse<sup>188</sup> intestine, Ezrin-mediated localization and regulation of membrane pumps is thought to direct lumen formation. But how would PTPN14-mediated regulation of E-cadherin alter initial lumen formation? One possibility arises from our observation of E-cadherin localization in 3D cultures of 2-cell Caco cysts. The bridge of E-cadherin observed in control cells suggests that E-cadherin-mediated adhesion is necessary for lumen establishment. Perhaps local regulation of E-cadherin adhesion enables surrounding cells to respond to the increased fluid pressure that results from activation of membrane pumps. Indeed, we observe a concentration of actin with this E-cadherin bridge; this concentration could stabilize the luminal surface in response to increased fluidic pressure in the developing lumen. The discovery and characterization of signals that trigger reorganization of E-cadherin from this luminal surface could provide new insight into functions of E-cadherin in non-junctional regions of the cell. Indeed, there are likely many

additional pathways and molecules that regulate the fine-tuning of cell adhesion and polarity—  
both in normal and pathogenic settings.

## **CHAPTER 4.**

### **PTPN14 PROMOTES MESENCHYMAL PHENOTYPES BY DESTABILIZATION OF THE ADHERENS JUNCTION**



## Introduction

More than 90% of cancer related deaths are associated directly to metastasis – the spread of tumor cells to distant organs where they establish secondary colonies<sup>189,190</sup>. A key part of the metastatic cascade is the cellular process called epithelial-mesenchymal transition (EMT), which increases the migratory and invasive potential of tumor cells by the loss of cell polarity and cell-cell adhesion<sup>4,191</sup>. Accordingly, the loss of these essential epithelial characteristics also contributes to chemotherapy resistance because of a hypothesized cancer stem cell (CSC) population that retains EMT features<sup>192</sup>. This remains a significant clinical issue because, after treatment, the CSC population in residual tumors aids in chemotherapy resistance and ultimately tumor recurrence<sup>193</sup>. Recently, PTPN14 has been implicated in the direct and indirect regulation of signaling pathways, including the TGF $\beta$ <sup>65</sup> and Hippo<sup>71-73</sup> pathways, which are known to induce EMT in a number of cell lines and tissues.

TGF $\beta$  is a secreted growth factor that interacts with and activates transmembrane serine/threonine receptors<sup>194</sup>. In canonical signaling, the type I receptors phosphorylate and activate Smad2/3 that form a complex with Smad4 to shuttle to the nucleus and regulate transcription of target genes<sup>195</sup>. Non-canonical (non-Smad) TGF $\beta$  signaling pathways include MAPK, Rho-like GTPase and the PI3K/AKT pathways. These non-canonical pathways can have cross-talk with canonical TGF $\beta$  signaling to elicit diverse responses from the TGF $\beta$  receptors<sup>196</sup>. Increased TGF $\beta$  signaling was first shown to induce EMT in a cell culture model whereby epithelial cells took on morphological changes from a cuboidal to a spindle-cell shape<sup>194</sup>. This morphological change was accompanied by a decrease of the epithelial marker, E-cadherin, and an increase in the EMT markers, vimentin and fibronectin<sup>194</sup>. PTPN14 over-expression is shown to induce EMT by increasing canonical TGF $\beta$  signaling through Smad4 in MDCK cells<sup>65</sup>. *In*

*in vivo* studies demonstrate that PTPN14 knockdown in the zebrafish embryo causes loss of TGF $\beta$ <sub>3</sub> expression in regions of the developing brain and heart leading to defects in organogenesis<sup>65</sup>. These studies demonstrate potential tumor suppressive and oncogenic functions of PTPN14 via the TGF $\beta$  pathway, although it is not known whether these are phosphatase-dependent or independent functions.

In addition to TGF $\beta$  signaling, PTPN14 directly interacts with and negatively regulates Yes-associated protein (YAP), a core component of the Hippo signaling pathway, in a cell density-dependent manner<sup>71-73</sup>. First discovered in *Drosophila* the Hippo pathway contains a core kinase cascade composed of Mst1/2 (Hippo) and Lats1/2 that dictates the phosphorylation state of the transcriptional co-activator, YAP<sup>197</sup>. Phosphorylated YAP is sequestered in the cytoplasm, but nonphosphorylated YAP can enter the nucleus to activate transcription of target genes. Multiple studies show that Hippo signaling is not regulated by a set of ligands and receptors, but rather by multiple upstream components that feed into the core kinase cascade<sup>198</sup>, most of which have roles in modulating cell polarity and adhesion<sup>199,200</sup>. The Hippo pathway has been implicated in inducing EMT<sup>74</sup> and mediating contact inhibition of proliferation<sup>201-204</sup>. Reports highlight a role for the AJ in regulating YAP localization through knockdown of the AJ proteins, E-cadherin,  $\alpha$ -catenin and  $\beta$ -catenin, which causes an increase in cell proliferation and nuclear YAP<sup>201,205</sup>. Our previous data identifying a role for PTPN14 in the regulation of E-cadherin membrane mobility (see Chapter 3) may provide a cue for YAP nuclear entry or sequestration in the cytoplasm. In most cases, cytoplasmic retention of YAP is mediated through the downstream kinase LATS1. It is reported that PTPN14 interacts with LATS1 and the upstream Hippo component, Kibra, to regulate the localization of YAP although PTPN14 can interact with LATS1 in a Kibra-independent manner<sup>206</sup>. However, reduced PTPN14 causes aberrant acinar formation in mammary epithelial cells (MCF10A), which is rescued by

exogenous expression of Kibra, indicating a cross-talk mechanism to control 3D morphogenesis<sup>206</sup>. In this context, the YAP transcriptional targets, CTGF and CYR61, were increased suggesting that knockdown of PTPN14 reinforces the oncogenic function of YAP<sup>206</sup>. Although efforts focus on understanding the effects of the Hippo signaling pathway in cell proliferation and contact inhibition, its role in EMT remains unclear. A pathway implicated in regulating EMT that also has roles in maintaining cell proliferation and differentiation is the Notch signaling pathway.

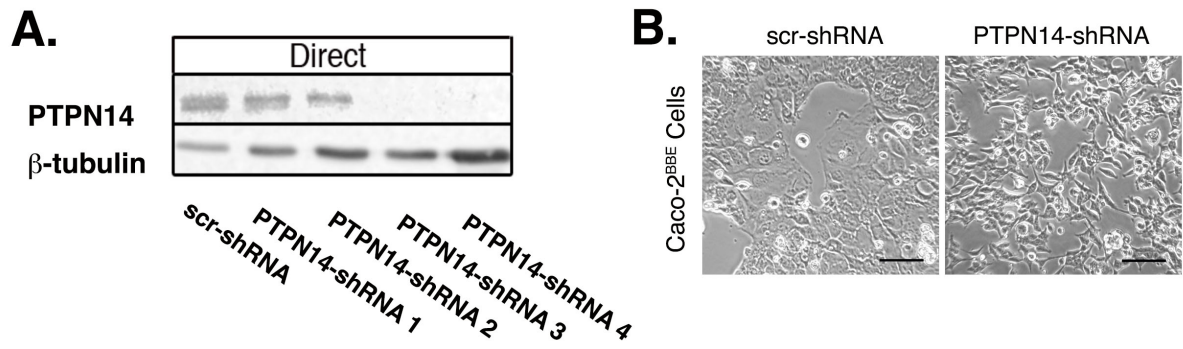
The Notch signaling pathway is evolutionarily conserved and is linked to cell proliferation<sup>207</sup>, apoptosis<sup>208</sup> and differentiation<sup>209,210</sup>. Notch signaling is carried out by direct cell-cell communication where ligands are expressed at the cell membrane to activate receptors on a neighboring cell<sup>211</sup>. Canonical Notch signaling is characterized by a series of proteolytic cleavages that prompt the intracellular domain of the Notch receptor to translocate to the nucleus and associate with the nuclear effector, CSL (CBF1, Suppressor of Hairless, Lag-1) to activate transcription of downstream targets (Reviewed in<sup>212</sup>). Aberrant Notch signaling is reported in tumorigenesis where it exhibits anti-proliferative and oncogenic functions in a context-dependent manner<sup>213,214</sup>. Importantly, Notch activation results in acquisition of mesenchymal characteristics reportedly through the upregulation of the E-cadherin repressors, Snail1<sup>215,216</sup> and Snail2/Slug<sup>217,218</sup>. The Notch pathway has also been reported to control cell morphogenesis via AJ remodeling in *Drosophila* ovarian follicles<sup>219</sup>. Similarly, our observations indicate that PTPN14 localizes to the AJ during early junction development and stabilizes the E-cadherin/ $\beta$ -catenin complex to maintain cell contacts (see chapter 3). Although it is not known whether PTPN14 is directly connected to Notch signaling, reports identify the interaction of PTPN14 with VEGFR3<sup>220</sup> and postulate a cross-talk between VEGFR3 and Notch signaling in endothelial cells to control proliferation and differentiation<sup>221,222</sup>. However, it remains unclear whether

Notch signals to PTPN14 or PTPN14 regulates a Notch response in cells to orchestrate cellular movements and/or maintain cell contacts. Here we continue our focus on PTPN14 regulation of AJ-mediated adhesion and show that decreased PTPN14 activity over time induces a specific EMT response through increased Notch signaling and the Snail2/Slug transcription factor.

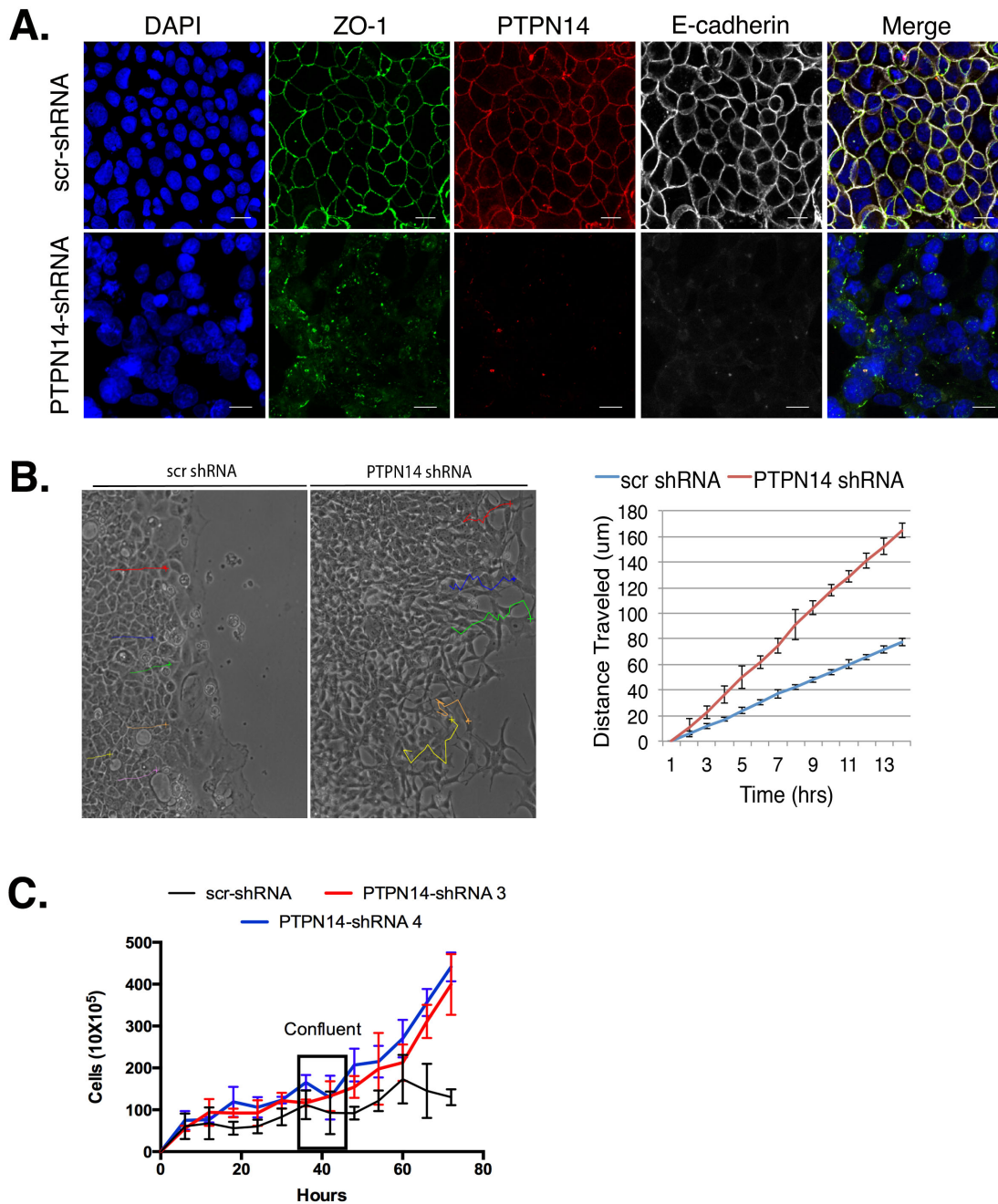
## **Results**

### **Decreased PTPN14 induces mesenchymal features in epithelial cells**

There is significant down regulation of E-cadherin expression in CRC<sup>223</sup>, which also appears to be a critical step in the process of EMT<sup>224</sup>. With the observations that PTPN14 is crucial for AJ-mediated adhesion and epithelial morphogenesis by regulating E-cadherin membrane movement (see chapter 3), we sought to determine if alterations in signaling occur over time in cells with depleted PTPN14. To do this, we infected Caco-2 cells with lentivirus expressing shRNA constructs targeting PTPN14. Following confirmation of decreased PTPN14 levels with two independent shRNA constructs, Caco-2 cells with decreased PTPN14 (Figure 15A) exhibited a change in morphology where the cells took on a more mesenchymal phenotype as compared to control cells that display a cobblestone-like morphology (Figure 15B). Since cell junctions are important for maintaining cell adhesion, we analyzed cell junction formation in confluent cultures. PTPN14 knockdown cells exhibited diminished levels of PTPN14 and E-cadherin with disrupted ZO-1 membrane staining as compared to control (scr) cells (Figure 16A). Since we observe diminished levels of junction proteins and cell migration is dependent on the stability of cell:cell contacts, we wanted to determine whether these defects in AJ and TJ proteins by knockdown of PTPN14 increases the mobility of cells and their directional motion. In order to address this, we performed a scratch assay and captured cell movement by live-cell imaging. By



**Figure 15. Decreased PTPN14 alters epithelial cell morphology.** **A)** Direct Western analysis indicates knockdown of PTPN14 via two separate shRNA-mediated knockdown constructs of PTPN14 (PTPN14-shRNA) in Caco-2<sup>BBE</sup> cells. **B)** Phase-contrast microscopy image of control (scr) and PTPN14 knockdown cells. Decreased PTPN14 causes cells to change from a classic cobblestone appearance to a spindle-cell morphology. Scale bars, 0.5mm



**Figure 16. Decreased PTPN14 induces mesenchymal features in epithelial cells.**

**A)** Localization of ZO-1 (green), PTPN14 (red) and E-cadherin (white) in control (scr) and cells exposed to PTPN14 knockdown over an extended amount of time (PTPN14-shRNA). Cells with decreased PTPN14 activity over an extended amount of time exhibit mis-localized and reduced ZO-1 and E-cadherin, respectively. Scale bars, 20 $\mu$ m. **B)** Scratch assay tracking individual cell movement over time. PTPN14 knockdown cells exhibit loss of collective cell migration and travel at double the velocity of control cells. Values equal mean (n=12)  $\pm$  SD. **C)** Growth curve assay with indicating that cells with decreased PTPN14 (PTPN14-shRNA) lose contact inhibition of proliferation. Values equal mean (n=2)  $\pm$  SD

tracking individual cells we observed that control cells have directional collective cell movement while PTPN14 knockdown cells have loss of this directional movement, increased velocity and have a scattered pattern of movement (Figure 16B). These data indicate that PTPN14 knockdown cells lack directional collective cell migration and have increased speed of migration (Figure 16B). We next wanted to determine if the increased mobility of cells and lack of directional cell migration globally affects the ability of cells to respond to cell-cell signaling by stopping proliferation when they come in contact with one another, a phenomenon known as contact inhibition of proliferation (Reviewed in <sup>225</sup>). To do this, we performed a growth curve assay where cells were plated at the same density and counted every six hours for up to 48 hours post-confluency. We found that, as compared to control cells that stopped proliferating once the cells came in contact with one another, PTPN14 knockdown cells do not undergo contact inhibition and thus, cells continue to proliferate (Figure 16C). This loss of contact inhibition of proliferation is a common characteristic of tumor cells to regulate tumor growth<sup>226</sup>.

### **Cells with diminished PTPN14 activity express markers of EMT**

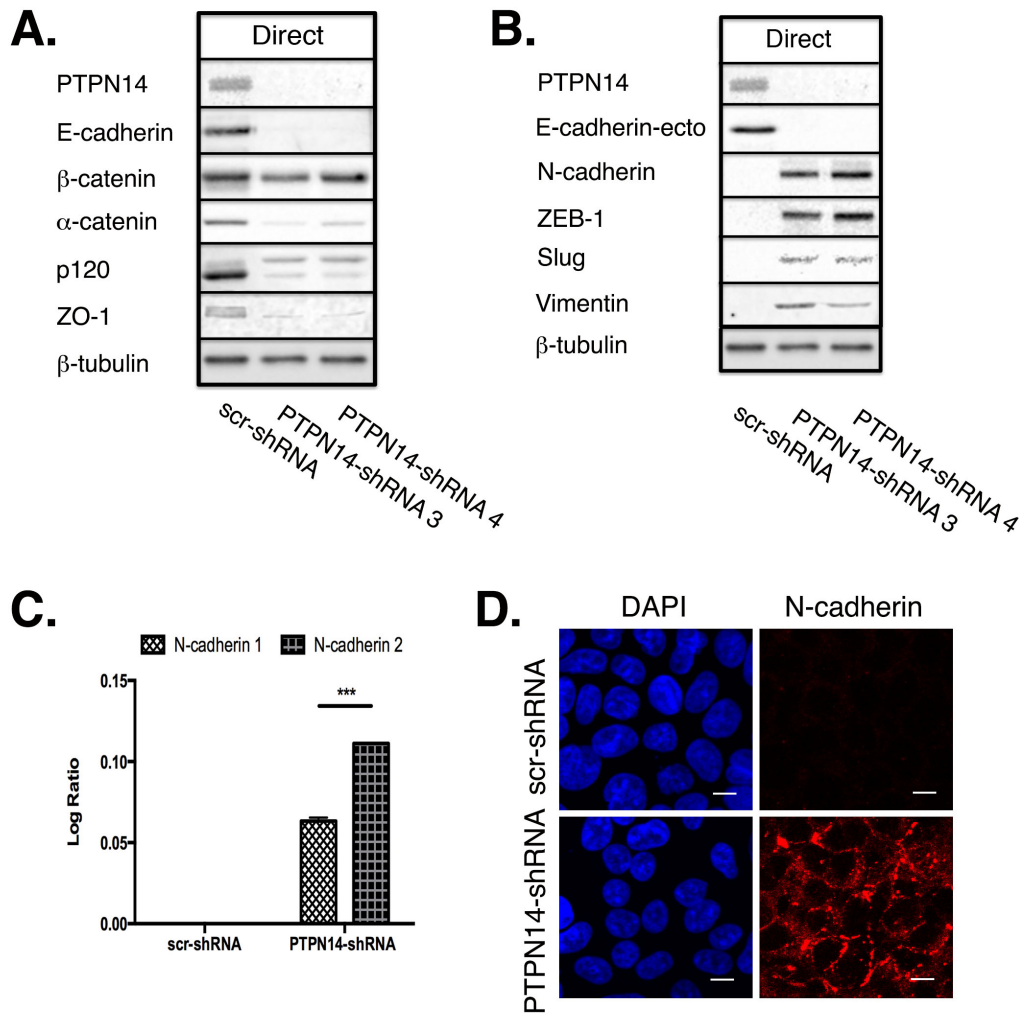
Our observation of diminished levels of membrane associated E-cadherin in cells depleted of PTPN14 suggested we could be altering E-cadherin protein at either a post-translational or transcriptional level. We wanted to determine if levels of total E-cadherin protein levels is altered in PTPN14-shRNA cells. Direct Westerns show no detectable E-cadherin in cells with decreased PTPN14 and the levels of the AJ proteins,  $\alpha$ -catenin and p120, and the TJ protein, ZO-1, are reduced with decreased PTPN14 while  $\beta$ -catenin levels remain the same (Figure 17A). The loss of E-cadherin is a molecular trademark of epithelial to mesenchymal transition (EMT), an event that is correlated with more migratory and invasive characteristics in tumor cells<sup>227,228</sup>. There is evidence that E-cadherin transcription is inhibited in EMT by the

transcription factors ZEB1<sup>229-231</sup>, Snail1<sup>223,232-235</sup>, Snail2/Slug<sup>233,236</sup> and Twist<sup>223,234</sup>. In addition to down regulation of epithelial markers, EMT is also distinguished by the presence of markers such as N-cadherin and Vimentin<sup>231</sup>. Together with the increase in cell migration and loss of E-cadherin, cells with decreased PTPN14 also express the EMT markers N-cadherin, Vimentin and the E-cadherin transcriptional repressors ZEB-1 and Snail2/Slug (Figure 17B). The expression of N-cadherin at the membrane is similar to what is observed during primitive streak ingression in normal development when “cadherin switching” occurs<sup>237,238</sup> although this can happen in tumor cells that have undergone a transition to mesenchymal characteristics<sup>239-241</sup>. Decreased PTPN14 causes increased N-cadherin transcription (Figure 17C) and displays membrane localization similar to E-cadherin in control cells (Figure 17D). This data indicates that cells with decreased PTPN14 exhibit a potential cadherin switch from E-cadherin to N-cadherin at the cell membrane potentially playing a role in altered cell adhesion and increased cell migration.

### **Knockdown of PTPN14 promotes a specific EMT response over time**

We wanted to determine when E-cadherin expression was reduced in cells with decreased PTPN14. Through quantitative RT-PCR, we looked at E-cadherin transcription levels in cells that were in culture for an extended amount of time with decreased PTPN14 (middle passage) as well as cells that were exposed to PTPN14-shRNA for a short amount of time (early passage). We found a step-wise decrease in E-cadherin the longer cells were in culture with decreased PTPN14 activity indicating that PTPN14 loss plays a pivotal role in the regulation of E-cadherin transcription (Figure 18A). A high percentage of tumor cells that arise from multiple tissues exhibit reduced E-cadherin transcription often time with a concomitant increase in the E-



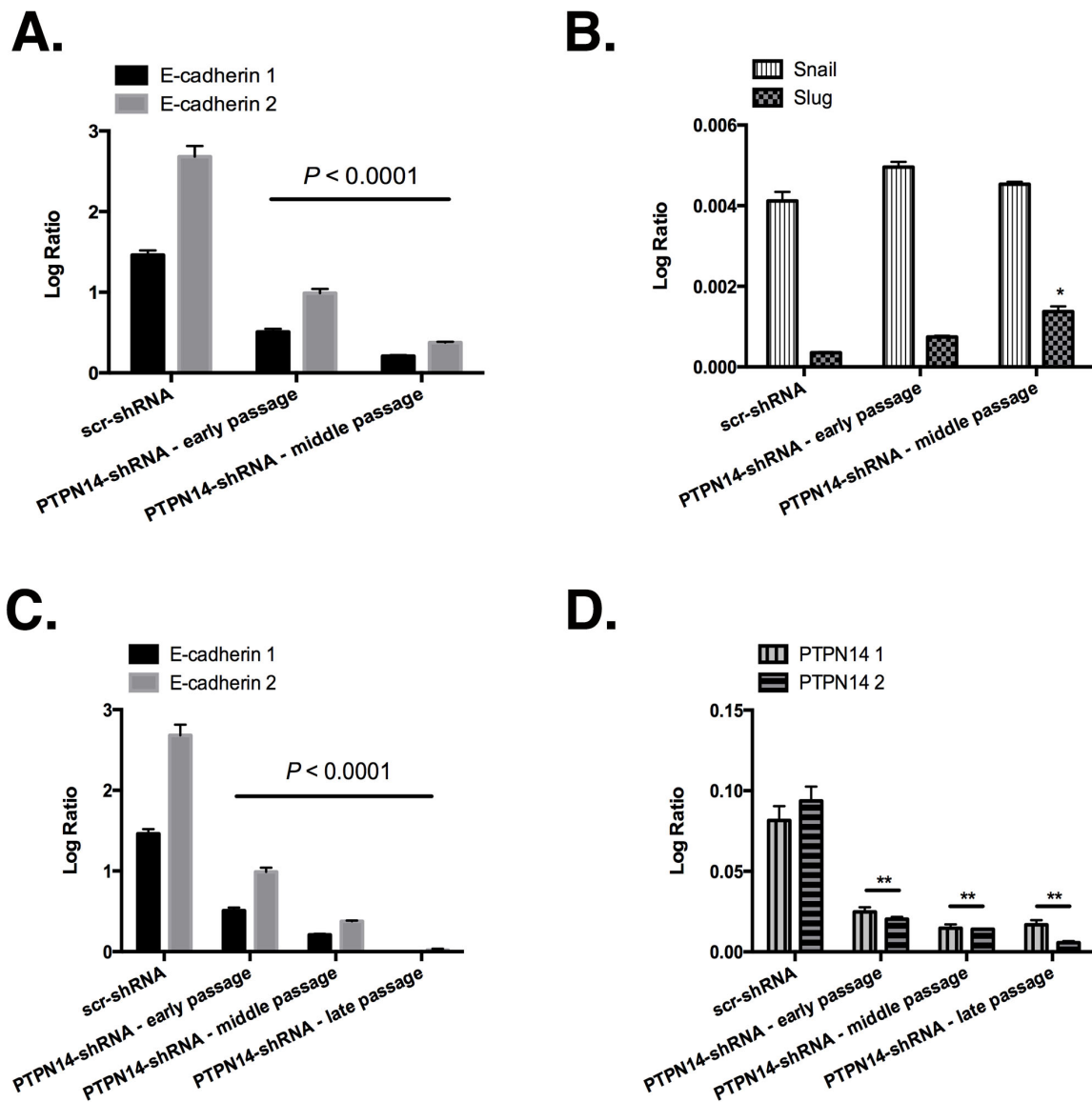


**Figure 17. Cells with diminished PTPN14 activity express EMT markers.** **A)** Direct Western analysis of AJ and TJ proteins in scr- and PTPN14-shRNA Caco-2 cells. AJ and TJ protein levels are decreased in cells with decreased PTPN14. **B)** Direct Western profile of EMT markers showing expression of N-cadherin, Vimentin and the E-cadherin repressors ZEB-1 and Snail2/Slug. **C)** Quantitative RT-PCR using two independent probes demonstrating a significant increase in N-cadherin transcription in PTPN14-shRNA cells. Values equal mean (n=2) +/- SD (\*\*\*)= $p < 0.001$ . **D)** Immunofluorescence staining showing N-cadherin is expressed and localized at the membrane in PTPN14-shRNA cells. Scale bars, 10μm.

cadherin repressors, Snail1, Snail2/Slug, ZEB-1 and Twist (Reviewed in <sup>242</sup>). Most notably, the E-cadherin repressor Snail2/Slug, serves as a potential predictive biomarker for metastasis and poor prognosis in CRC<sup>243</sup>. Along with a step-wise decrease in E-cadherin we see a step-wise increase in Slug transcription, suggesting that PTPN14 directly or indirectly regulates the transcription of Slug to induce EMT characteristics (Figure 18B). We examined E-cadherin transcription in cells with longer exposure to decreased PTPN14 (late passage) and found that there was no detectable E-cadherin transcription while knockdown of PTPN14 remained the same (Figures 18C and D). This suggests that prolonged decreased PTPN14 causes an EMT response over time specifically through the Snail2/Slug transcription factor.

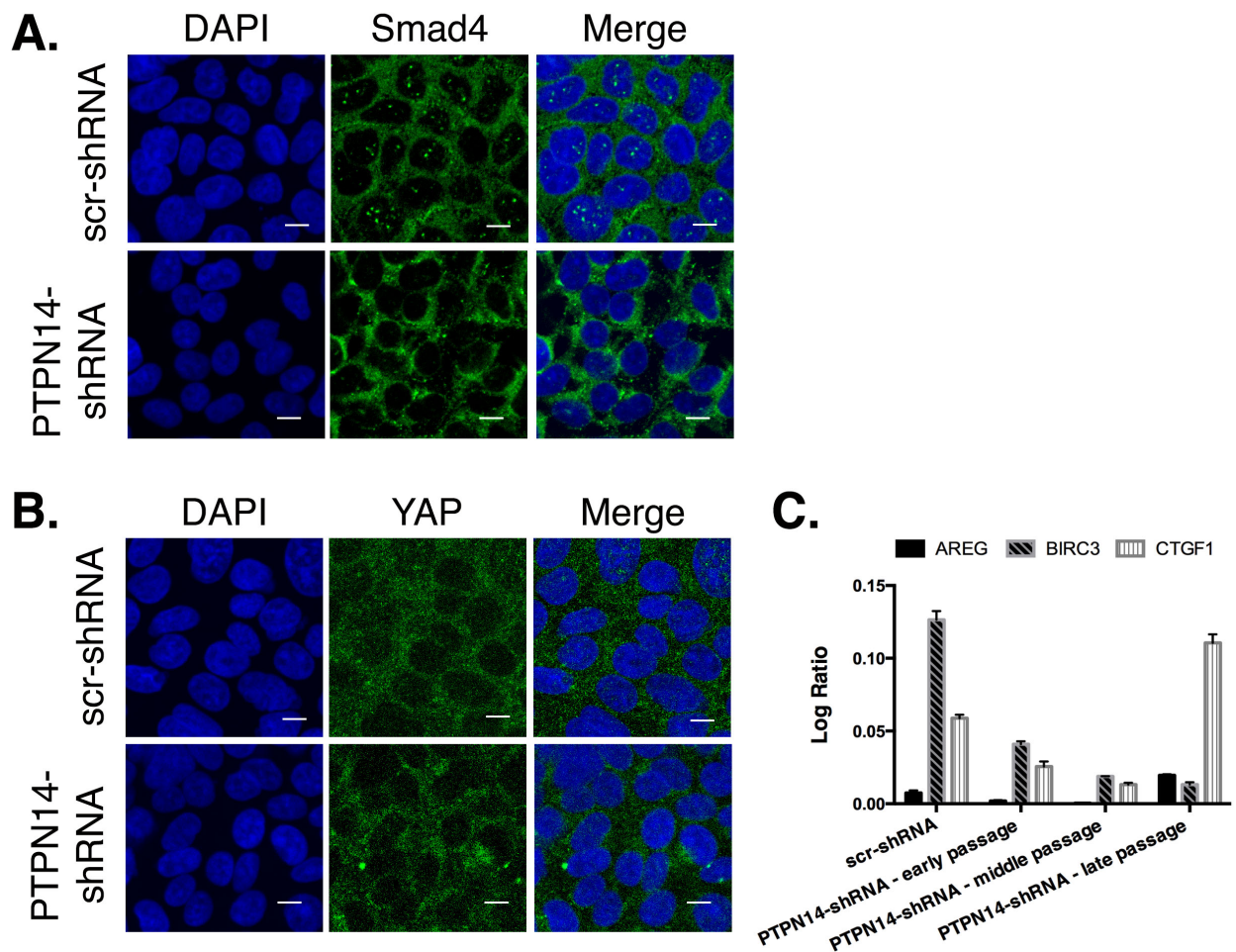
#### **Decreased PTPN14 activity triggers Notch signaling**

To determine if induction of EMT characteristics in PTPN14 knockdown cells were due to alterations of pathways that are regulated by PTPN14, we examined the targets of the TGF $\beta$  and Hippo pathways. PTPN14 is implicated in controlling TGF $\beta$  signaling through increased nuclear translocation of the downstream signaling effector Smad4 in canine MDCK cells<sup>65</sup>. In control cells, Smad4 remained predominantly cytoplasmic (Figure 19A, top panel). In addition, PTPN14 knockdown cells also had cytoplasmic Smad4 (Figure 19A, bottom panel), indicating that these cells do not have increased TGF $\beta$  signaling through Smad4. In addition, in mammary epithelial cells (MCF10A), PTPN14 negatively regulates the oncogenic function of the downstream pathway component, Yes-Associated protein (YAP)<sup>202</sup>. When Hippo signaling is deregulated, YAP enters the nucleus and subsequently triggers activation of target genes



**Figure 18. Knockdown of PTPN14 promotes a specific EMT response over time.**

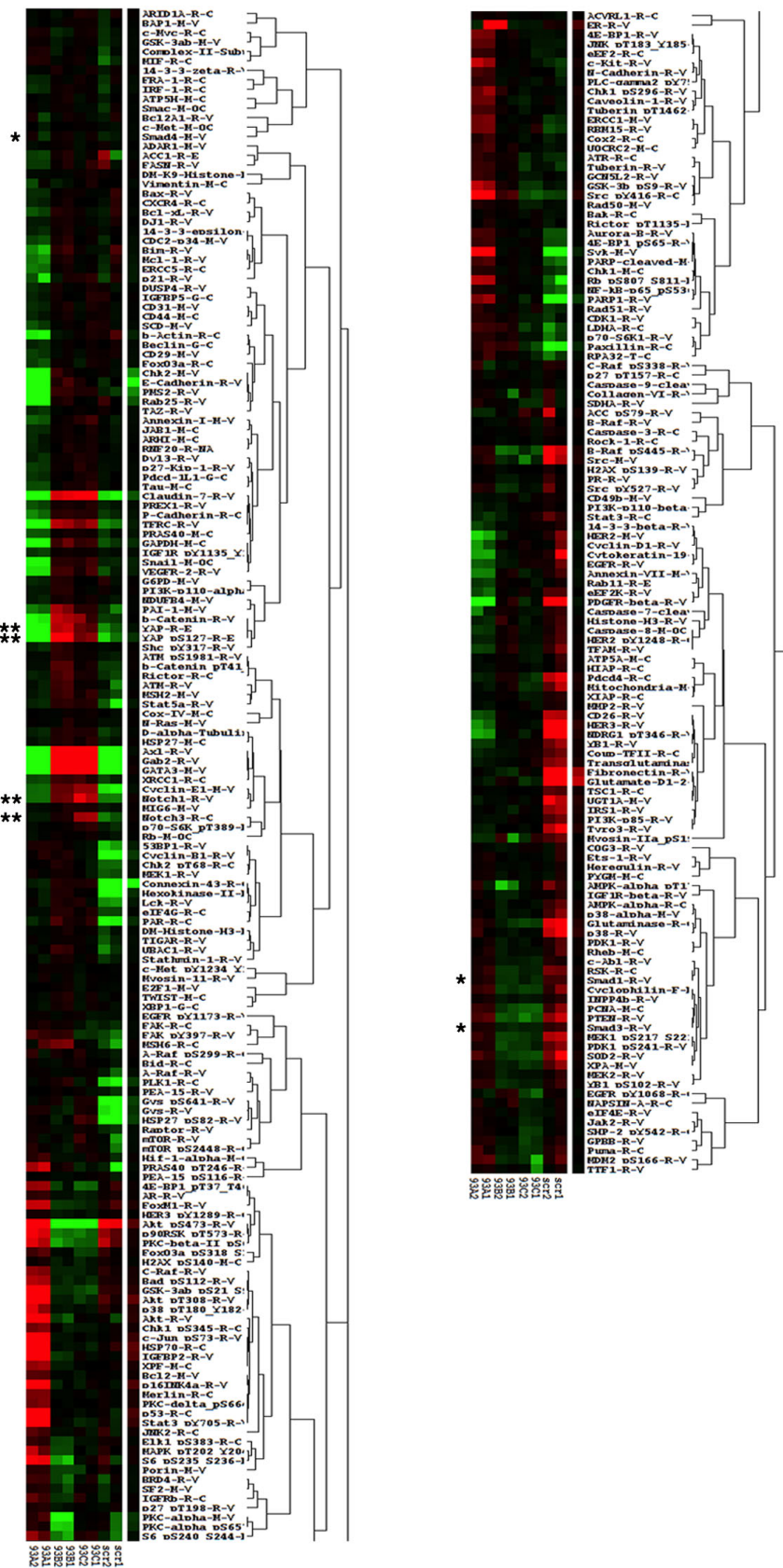
**A)** Quantitative RT-PCR using two independent probes shows a significance step-wise decrease in E-cadherin transcription in PTPN14-shRNA early and middle passage cells. Values equal mean (n=3) +/- SD. **B)** Comparison of Snail and Slug transcription by qRT-PCR in PTPN14 knockdown cells. Slug transcription exhibits a step-wise increase over time with exposure to decreased PTPN14 activity while Snail transcription does not change. Values equal mean (n=2) +/- SD (\*=p<0.05). **C)** E-cadherin transcription by qRT-PCR using two independent probes in PTPN14 knockdown cells. A late passage of E-cadherin shows virtually no transcription suggesting that E-cadherin is being transcriptionally repressed over time while PTPN14 transcription in **D)** remains the same. Values equal mean (n=3) +/- SD (\*\*=p<0.005).



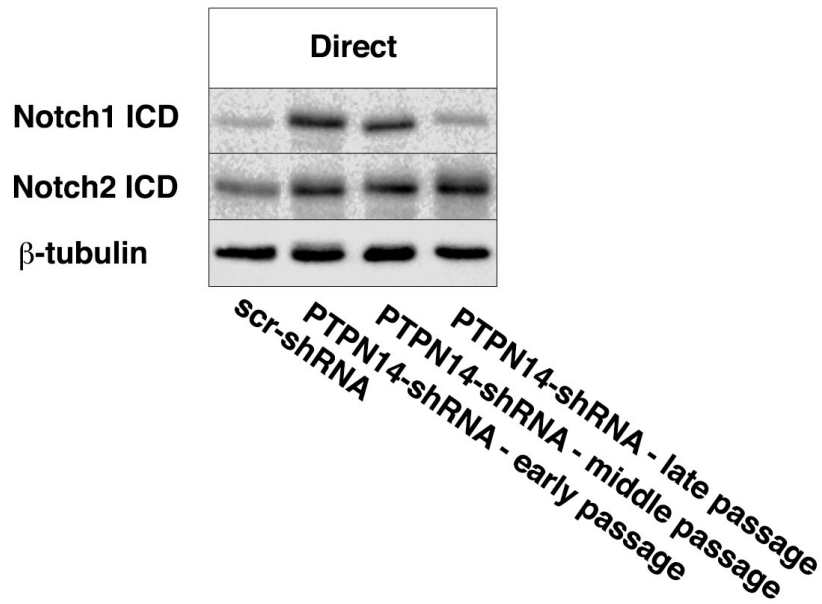
**Figure 19. EMT in cells with decreased PTPN14 is independent of TGF $\beta$  and Hippo signaling.** **A)** Staining with total YAP antibody reveals that YAP is cytoplasmic in both control (scr) and PTPN14 knockdown confluent cells. These data indicate no increased nuclear YAP, therefore proposing a YAP-independent function of PTPN14 in Caco-2 cells. Scale bars, 10 $\mu$ m. **B)** Smad4 staining is cytoplasmic in both control (scr) and PTPN14-shRNA cells, indicating no increased translocation of Smad4 to the nucleus and subsequent increase in TGF $\beta$  signaling in PTPN14 knockdown cells. Scale bars, 10 $\mu$ m. **C)** Quantitative RT-PCR probing for YAP targets, AREG, BIRC3 and CTGF1 in control and PTPN14 knockdown early, middle and late passages. Values equal mean (n=1)  $\pm$  SD.

(Reviewed in <sup>244</sup>). YAP localization did not change between control and PTPN14 knockdown cells (Figure 19B) and the YAP transcriptional targets; AREG, BIRC3 and CTGF1 did not appear to have any distinct changes to correlate with the EMT phenotype observed in cells with decreased PTPN14 (Figure 19C). These data suggest a YAP-independent function of PTPN14 in intestinal epithelial cells. Given that there is no change in pathways that are regulated by PTPN14, we utilized a non-biased approach and performed reverse phase protein array (RPPA) analysis on control (scr), early (93C), middle (93B) and late (93A) depleted PTPN14 cells (Figure 20). We confirmed our IF findings by observing no change in TGF $\beta$  signaling by Smad1, 3 and 4. Similar to what we saw by IF, we saw an increase in total YAP, but with an increase in phospho-YAP-S127 that blocks YAP activation<sup>202</sup>. Lastly, we uncovered a step-wise increase in Notch1 over time when cells are exposed to lower levels of PTPN14 activity.

Canonical Notch signaling occurs when there is an extracellular interaction of a Notch transmembrane ligand with a Notch transmembrane receptor; this interaction initiates proteolytic cleavage of the Notch receptor and release of its intracellular domain (ICD) (Reviewed in <sup>212</sup>). Notch ICD translocates to the nucleus and interacts with CSL (CBF1/Suppressor of Hairless/LAG-1) to initiate the transcription of Notch target genes<sup>245</sup>. We probed confluent whole cell lysates with antibodies to detect Notch1 and Notch2 and observed a step-wise increase of both the Notch 1 and 2 ICD corresponding with an increase in the time the cells have been depleted of PTPN14 (Figure 21). The decreased adhesion of PTPN14 late depleted cells due to cadherin switching would likely prevent Notch signaling from increasing since the Notch signaling cascade is dependent on cell membrane bound receptors and ligand interactions. These data suggest a potential regulation of Notch signaling in cells with decreased PTPN14 activity.



**Figure 20. Reverse Phase Protein Array (RPPA) for Caco-2 cells of varying lengths of time with decreased PTPN14 activity.** Duplicate control (scr - right), early (93C), middle (93B) and late (93A - left) depleted cells were stained for 285 unique antibodies and were analyzed on Array-Pro then by supercurve Rx64 3.1.1. Samples were probed with the antibodies using a CSA amplification approach and visualized by DAB colorimetric reaction. The heatmap was generated in Cluster 3.0 as a hierarchical cluster using Pearson Correlation and shows overall patterns of protein levels. A green signal indicates a decrease in protein while a red signal indicates an increase in protein level. The RPPA Core Facility at MD Anderson completed all analyses. \* indicates Smad protein; \*\* marks YAP protein and \*\*\* designates Notch protein levels in cells with depletion of PTPN14. A step-wise increase of Notch was observed in cells with decreased PTPN14 over an extended amount of time.



**Figure 21. Decreased PTPN14 activity triggers an increase in Notch.** Direct Western analysis indicating an increase in Notch1 and 2 in PTPN14 knockdown cells. The increase in Notch1/2 is seen in early and middle passages of cells with reduced PTPN14 activity; however, Notch1 decreases when cells undergo an EMT-like phenotype, suggesting that Notch signaling may be involved in the induction of EMT in PTPN14 knockdown cells.



## Discussion

In recent years, our understanding of EMT has greatly increased yet many questions remain on the molecular mechanisms that govern the changes that occur to eventually cause epithelial tumors to acquire mesenchymal characteristics and metastasize. These data demonstrate that over an extended amount of time, decreased activity of the tyrosine phosphatase, PTPN14, causes an EMT response in cultured epithelial cells. Our previous findings illustrate a role for PTPN14 in the stabilization of AJ-mediated adhesion through maintenance of  $\beta$ -catenin-Y654 phosphorylation (See Chapter 3). It is well known that cell:cell adhesion and polarity orchestrate a highly regulated EMT process in normal developmental<sup>4,246</sup>. However, cancer cells hijack this biological process to acquire more migratory and invasive characteristics<sup>247</sup>, making the pathological process of tumor EMT quite difficult to delineate. By examining cells with varying lengths of time with decreased PTPN14 activity, we identified a step-wise decrease in E-cadherin transcription coupled with increase of the Snail2/Slug transcription factor while decreased PTPN14 transcript levels remained the same. This highlights a potential tumor suppressor function of PTPN14 that primes cells to undergo EMT. However, whether or not additional alterations occur to cause this transition is yet to be determined. Since PTPN14 overexpression is shown to induce EMT through regulation of TGF $\beta$  signaling in MDCK cells<sup>65</sup>, we saw this as a candidate pathway, however we did not observe any increase in TGF $\beta$  signaling by Smad4 or Smad3 (data not shown) translocation to the nucleus. Also, PTPN14 is implicated in Hippo signaling and subsequent YAP-dependent migration in mammary epithelial cells<sup>72</sup>, but we did not observe any nuclear YAP in cells that underwent EMT. This was an interesting find since cells with decreased PTPN14 exhibit loss of contact inhibition of proliferation and studies suggest that proliferating cells normally exhibit nuclear YAP<sup>202</sup>. From this, our data suggests that PTPN14 may have a cell-specific effect on signaling in intestinal

epithelial cells independent of TGF $\beta$  and Hippo signaling. One proposed mechanism from our work is that PTPN14 may be regulating Notch signaling.

Notch signaling is an evolutionarily conserved pathway<sup>248</sup> that mediates cell survival<sup>249</sup> and cell-fate determination during development<sup>209</sup>. We see a step-wise increase over time of Notch1 and Notch2 in cells with decreased PTPN14. Reports identify that Snail2/Slug is a direct target of Notch in cardiac endothelial cells to induce EMT<sup>217</sup>. The step-wise increase we see of Notch1 and 2 coupled with the increase in Snail2/Slug transcription begs the question of whether PTPN14 alone is responsible for the upregulation of Notch signaling to elicit a conversion of epithelial cells to acquire mesenchymal characteristics. These questions remain unanswered at this time, however the identification that decreased PTPN14 activity as a key factor in tumor progression makes it a potential target for therapeutics in cancer treatment.

## **CHAPTER 5.**

DEFINING THE FUNCTION OF THE *NF2* TUMOR SUPPRESSOR PROTEIN, MERLIN,  
IN COORDINATING CELL ADHESION AND POLARITY

## Introduction

Development of epithelial cell junctions involves a process where an immature junction containing both the AJ and TJ proteins transitions into distinct polarized AJ and TJ structures with the final placement of the TJ just apical of the AJ<sup>181,250,251</sup>. Work has revealed that the protein product of the *Nf2* tumor suppressor gene, Merlin, coordinates cell adhesion and polarity of epithelial cells by directly linking the apical polarity protein, Par3, to the AJ protein,  $\alpha$ -catenin. This physical interaction generating a junctional polarity complex is required in the early stages of epithelial junction formation<sup>84</sup>. Merlin is a member of the FERM family of proteins consisting of a highly structured N-terminal FERM domain and a C-terminal tail connected by a flexible linker region. Unique to Merlin is an unstructured N-terminal region consisting of 17 amino acids four of which are serines. Deletion of the first 17 amino acids of Merlin blocks Merlin tumor suppressor function and recruitment to the actin cytoskeleton<sup>98</sup>. Recruitment of Par3 and aPKC is required for the formation of TJs and the establishment of polarity<sup>100,108</sup>. Also, the N-terminal portion of Merlin is required for association of Par3 with the AJ complex<sup>84</sup>, which may be essential for the initial recruitment of aPKC and TJ proteins. Loss of Merlin causes mis-localization of aPKC away from the apical cell membrane and inhibits TJ formation<sup>84</sup>. When aPKC kinase activity is blocked, cell polarity is altered and formation of functional TJ is blocked<sup>100</sup>, suggesting that mis-localization of aPKC affects cell membrane organization. This indicates that loss of either aPKC or the N-terminal region of Merlin exhibit similar phenotypes<sup>98</sup>. This raises the possibility that aPKC may regulate Merlin-mediated cell polarity and junction formation through phosphorylation of the N-terminal region of Merlin.

Several lines of evidence suggest that Merlin can be post-translationally modified through phosphorylation at specific serine residues by multiple kinases<sup>76,252-254</sup>. Phosphorylation of Merlin at serine residue 518 by Pak regulates Merlin activity by causing a proposed intra-

molecular head-to-tail interaction, which causes inactivation of the protein and triggers cell proliferation<sup>255</sup>. Mammalian cell culture studies suggest that the closed form of Merlin is active and can inhibit proliferation<sup>256</sup> whilst studies in *Drosophila* indicate that the open form of Merlin contains all necessary genetic functions<sup>257,258</sup>. Currently, it is not known whether this distinction between flies and mammals represent any functional difference and whether phosphorylation of Merlin in either context is directly correlated with its role in junction formation and polarity. More specifically, it is believed that loss of Merlin in human diseases can occur due to phosphorylation and subsequent proteasomal degradation<sup>76,252,253,255,259,260</sup>. Phosphorylation of Merlin at threonine 230 and serine 315 by the AKT kinase, targets Merlin for ubiquitination and subsequent degradation<sup>259</sup>. Additionally, recent reports demonstrated that AKT phosphorylates Merlin at serine 10 that is increased upon S518 phosphorylation to enhance Merlin degradation<sup>252</sup>. Ultimately, loss of Merlin leads to loss of contact inhibition and proliferation<sup>256,261,262</sup>, properties that are utilized to differentiate between normal and cancerous cells making the understanding of Merlin post-translational modifications and potential effects on Merlin protein stability of increasing importance. Here we describe the function(s) of aPKC-mediated phosphorylation of Merlin in regulating cell adhesion and apicobasal polarity.

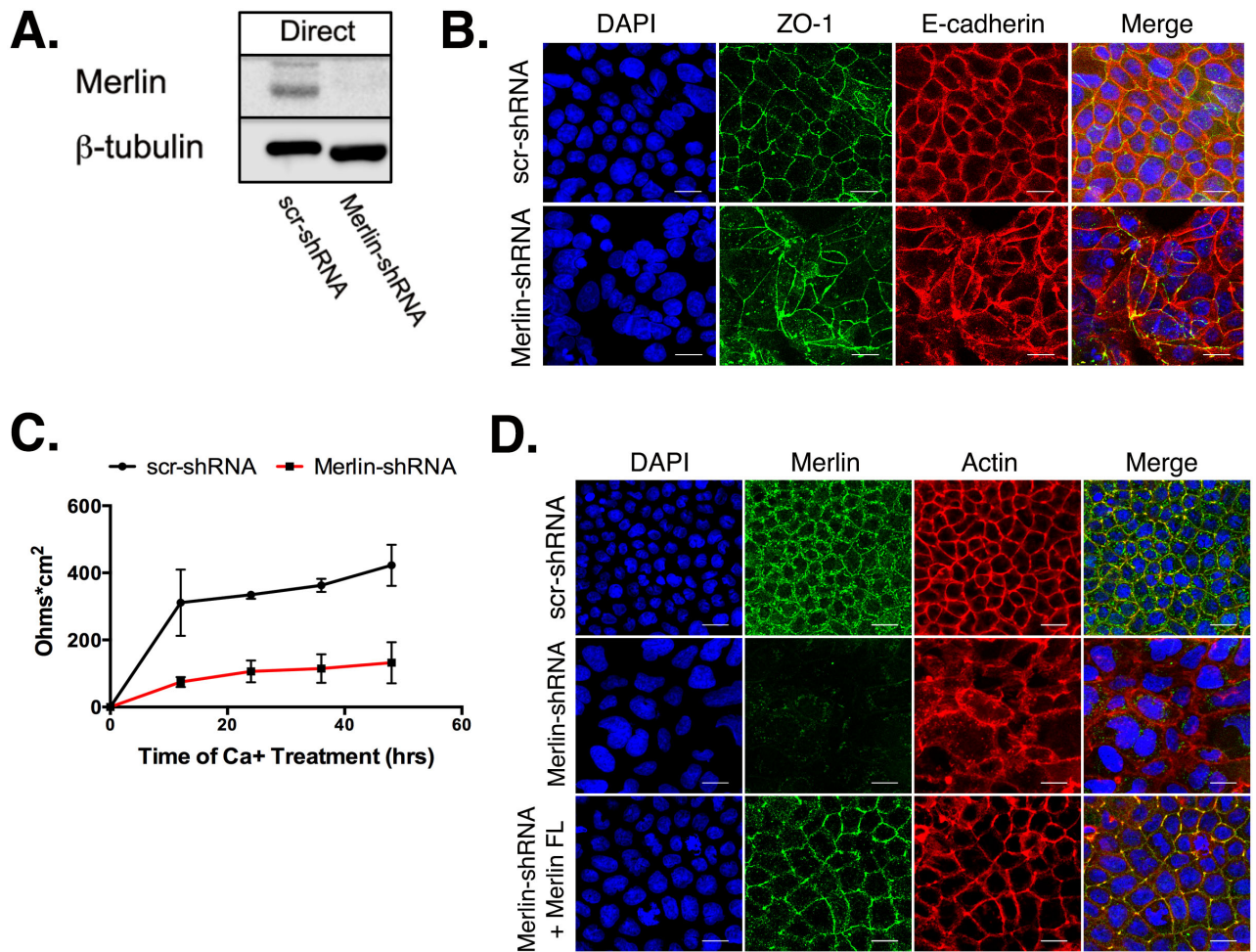
## **Results**

### **Merlin is required for the establishment of junctions in intestinal epithelial cells**

Previous work establishing a role for Merlin in the development of epithelial cell adhesion and polarity in the stratified epithelium of the epidermis suggested that Merlin linked cell polarity and AJ proteins during early junctional morphogenesis<sup>84</sup>. To determine if this role of Merlin occurred in a broad range of epithelial tissues or was specific to stratified epithelium we infected the simple epithelium intestinal cell line Caco-2 with shRNA lentivirus targeting Merlin.

We confirmed decreased levels of Merlin by direct Western blot (Figure 22A) and found that cells depleted of Merlin displayed disrupted TJs and AJs as shown by staining with the TJ marker ZO-1 and the AJ marker E-cadherin (Figure 22B). Given this alteration in junctional proteins, we wanted to determine whether there was also a disruption in the formation of functional TJs by performing trans-epithelial resistance (TER). Similar to what was previously observed in keratinocytes, TJ integrity is compromised in Merlin knockdown cells as revealed by reduced TER (Figure 22C), indicating that Merlin is required for the formation of functional TJs in numerous types of epithelial cells.

As a member of the ERM protein family, Merlin is known to convey signals from the surface membrane to the actin cytoskeleton, but how Merlin transmits these signals to the actin cytoskeleton remains unclear. Reports identify that Merlin can be involved in actin remodeling by association with actin regulatory proteins such as the WASP (Wiskott-Aldrich syndrome protein) family protein, N-WASP<sup>263</sup> and  $\alpha$ -catenin<sup>84</sup>. Also, the first 17 amino acids are required to place Merlin in an actin rich cellular compartment suggesting these amino acids may regulate how Merlin conveys signals to the actin cytoskeleton<sup>98</sup>. To determine if depletion of Merlin in simple columnar epithelial cells resulted in alterations of actin organization similar to what was observed in the skin, we looked at Merlin localization and actin in Caco cells with decreased Merlin. Decreased Merlin alters actin dynamics as compared to control (scr) cells (Figure 22D, middle panel). To determine if this was a direct result of decreased levels of Merlin, we expressed shRNA-resistant full-length Merlin. Expression of full-length Merlin led to actin organization at cell:cell contacts in Merlin knockdown cells (Figure 22D, bottom panel). This data indicates that Merlin is required for the establishment of epithelial junctions not only in a stratified epithelium, but also in the columnar epithelia of intestinal cells.



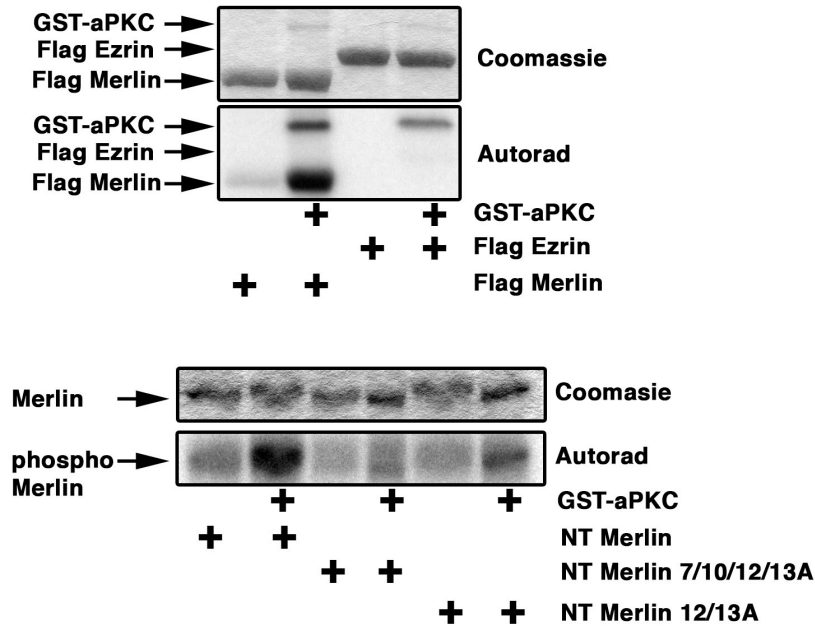
**Figure 22. Merlin is required for the establishment of AJs and TJs in Caco-2 cells.** **A)** Direct Western blot confirms knockdown of Merlin in Caco-2 intestinal epithelial cells. **B)** Loss of Merlin disrupts epithelial architecture as shown by staining with the TJ marker, ZO-1 (green), and the AJ marker, E-cadherin (red). **C)** TJ integrity is compromised in Merlin knockdown cells revealed by trans-epithelial resistance (TER). **D)** Merlin localization and actin organization is restored when full-length Merlin is expressed in Merlin knockdown cells.

## **The N-terminal region of Merlin is required for cell polarity and is phosphorylated by aPKC**

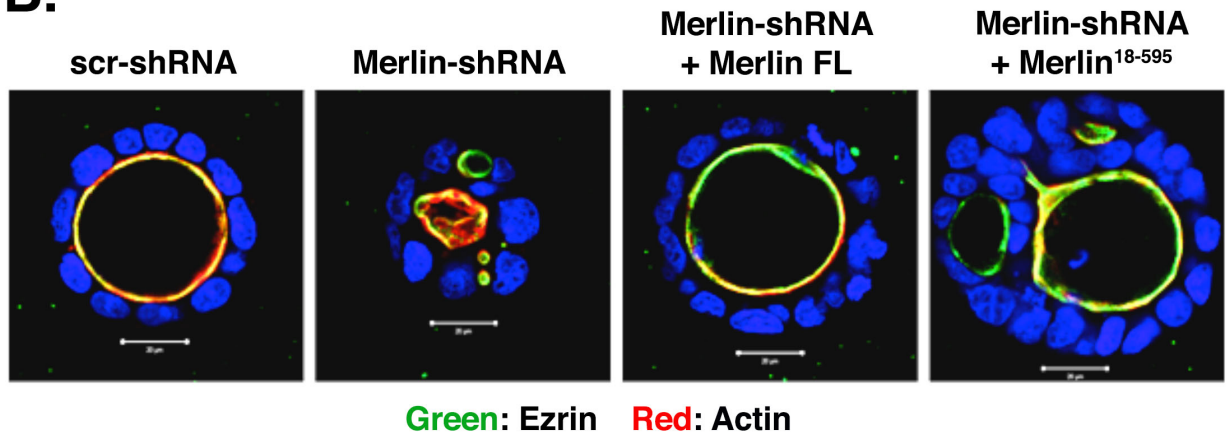
Inhibition of aPKC activity impairs functional TJ formation<sup>100</sup>, which is important for cell polarization and epithelial function. Given that loss of either aPKC or Merlin exhibit similar phenotypes and that Merlin could potentially be phosphorylated at any of the four serines in the first 17 amino acids, we hypothesize that aPKC may have a role in Merlin-mediated cell polarity and junction formation by post-translational modification of Merlin. To determine if aPKC phosphorylates the N-terminal portion of Merlin, we performed an *in vitro* kinase assay. aPKC phosphorylates Merlin and not the closely related ERM protein family member, Ezrin, that lacks the unique 17 amino acids of Merlin (Figure 23A). The candidate phosphorylation sites of the Merlin protein at serine residues 7, 10, 12 and 13 are unique among ERM proteins and mutation of all four serines within this region inhibit phosphorylation of Merlin by aPKC (Figure 23A). The N-terminal region of Merlin is required for TJ establishment through the tethering of the apical protein, Par3, to the AJ protein,  $\alpha$ -catenin; an association that must occur for the formation of functional junctions<sup>84</sup>. The formation of functional TJs is directly linked to cell polarity as TJs serve as a fence to restrict apical proteins to the apical membrane<sup>24-26</sup>. To identify whether the N-terminal region of Merlin is critical for cell polarity in Caco-2 cells, we performed a 3D culture cyst formation assay<sup>94,165,167</sup>. Compared to empty vector control, Merlin-depleted (Merlin-shRNA) cells present with a multiple lumen phenotype (Figure 23B). However, this phenotype is rescued when full-length Merlin (Merlin FL) is expressed in these cells (Figure 23B). A multiple lumen phenotype is seen when the same cells expressed a truncated form of Merlin lacking the first 17 amino acids (Merlin<sup>18-595</sup>) (Figure 23B, right panel), indicating that the N-terminal region is critical for the role of Merlin in cell polarity.



**A.**



**B.**

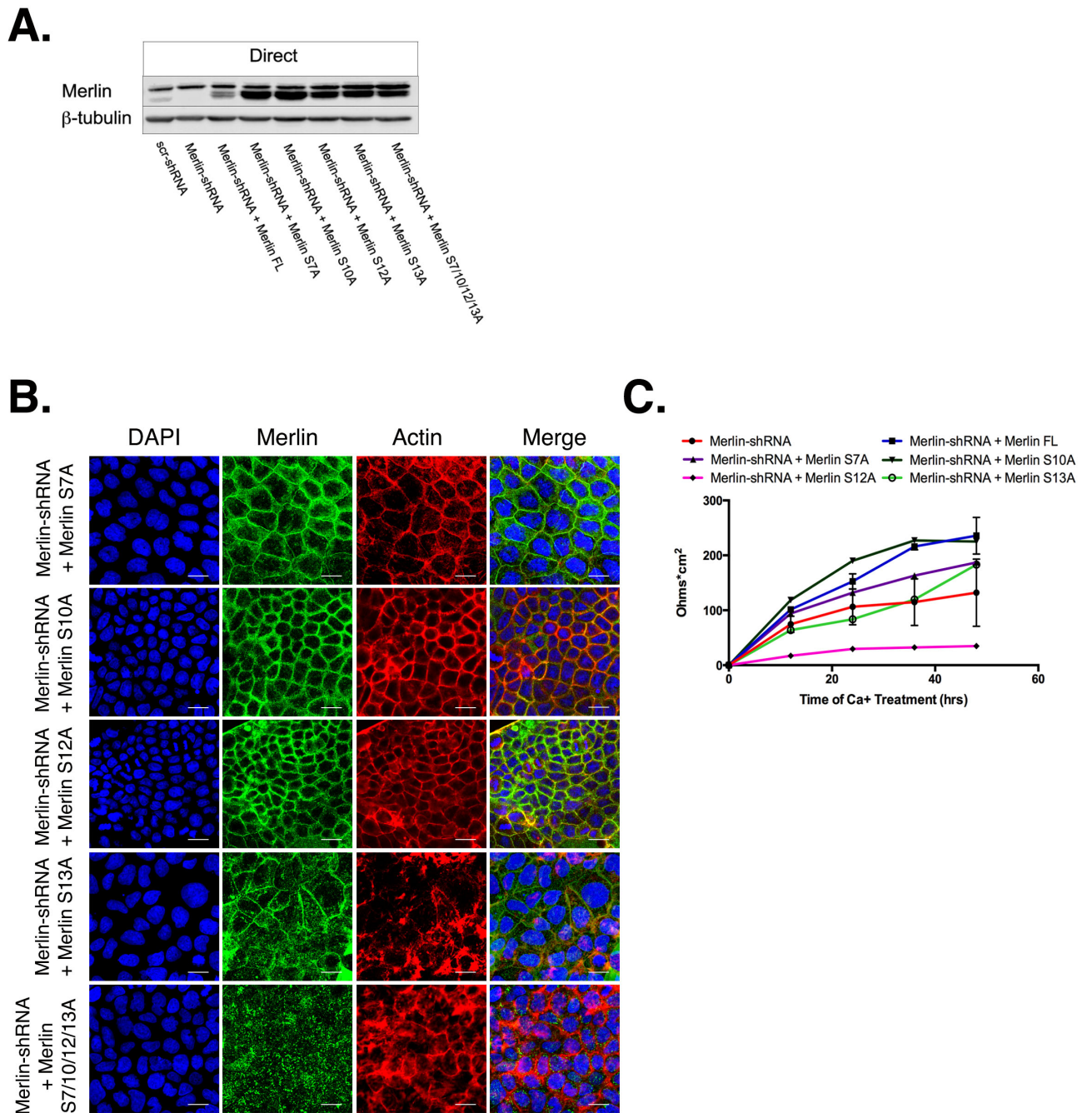


**Figure 23. The N-terminal region of Merlin is required for apicobasal polarity and is phosphorylated by aPKC. A)** In vitro kinase assay showing phosphorylation of Merlin by aPKC, but not the closely related ERM protein, Ezrin (top panel). Phosphorylation of Merlin is inhibited when non-phosphorylatable mutations are present at S7, S10, S12, and S13. Phosphorylation is reduced with mutations at S12 and S13 only (bottom panel). **B)** 3D lumen formation assay indicating that the extreme N-terminal region of Merlin is required for formation of a single central lumen.

### **aPKC regulates Merlin localization and function through post-translational modifications**

To determine the specific phosphorylation site(s) of Merlin by aPKC we mutated each individual serine site and stably expressed an shRNA-resistant mutant construct in Caco-2 cells that have Merlin knockdown (Figure 24A). Actin is disrupted in cells where Merlin is unable to be phosphorylated at residue S13 in a similar manner as cells with knockdown of endogenous Merlin (Figure 24B). This suggests that the phosphorylation event on residue S13 of Merlin may be necessary for proper loading of the junctional polarity complex ( $\alpha$ -catenin/Merlin/Par3) onto the AJ during junctional development. Also, phospho-deficient mutants of residues S7, S10, or S12 results in decreased junctional integrity as seen by TER (Figure 24C). This suggests that phosphorylation at these residues may be critical for the release of the aPKC/Par3 complex to the apical membrane for the formation of TJs. From this, we see that aPKC phosphorylation of Merlin can possibly alter the function of Merlin, an event that has the potential to cause interference with cell adhesion and promote Merlin-mediated tumorigenesis.

It is well documented that Merlin mediates contact inhibition of proliferation<sup>256,261,264-266</sup>, the ability of cells to stop proliferating when they come in contact with one another. Loss of contact inhibition can occur when tumor cells circumvent the innate mechanisms that cells use to control proliferation, such as growth control signals from neighboring cells. Several mechanisms exist for how Merlin regulates contact inhibition in cultured mammalian cells, including suppression of Rac recruitment to the plasma membrane<sup>261</sup> and through its interaction with the transmembrane receptor, CD44<sup>256</sup>. Loss of Merlin function contributes to tumor initiation and/or progression due to the inability of cells to establish stable AJs, which leads to the loss of contact inhibition. However, it is unknown whether phosphorylation of Merlin at its N-terminal region



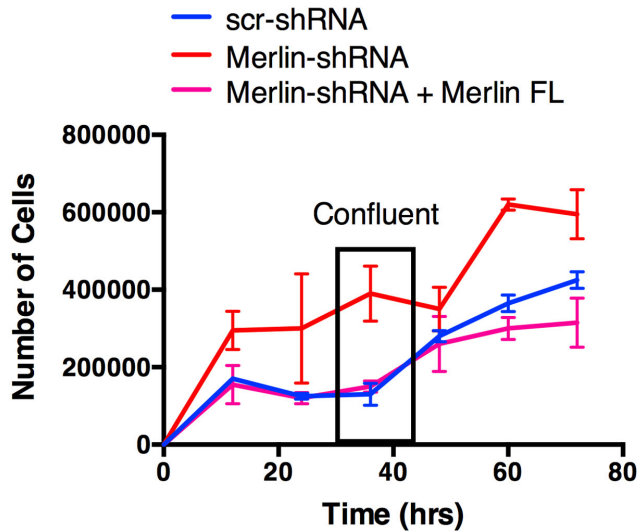
**Figure 24. aPKC regulates Merlin localization and function through post-translational modifications.** **A)** Western analysis of re-expression of Merlin point mutants in Caco-2 cells. **B)** Merlin (green) and actin (red) staining in cells expressing Merlin point mutants. Merlin localization and actin organization are altered in cells harboring a Merlin mutation at S13 and at all four serine sites in the N-terminal region of Merlin. **C)** TER readings for Merlin point mutants. TJ integrity is compromised in Merlin N-terminal mutations at S7, S12 and S13.

is critical for its role in mediating contact inhibition. To address the potential effect phosphorylation may have on Merlin-mediated contact inhibition, we performed a growth curve assay where cells were plated and counted every six hours up to confluency and post confluency. We identified that Merlin knockdown in Caco-2 cells did exhibit loss of contact-dependent inhibition of proliferation and expression of full-length sh-RNA resistant Merlin rescues the loss of contact inhibition (Figure 25A). We also observed a loss of contact inhibition when all phosphorylation is blocked at the four serines at residues S7, S10, S12, and S13 of the N-terminal region of Merlin (Figure 25B); this is similar to knockdown of Merlin. Loss of contact inhibition is observed when S12 and S13 are unable to be phosphorylated (Figure 25B), suggesting that these phosphorylation events may be critical for the stabilization of AJ-mediated adhesion by Merlin. Our evidence does not currently support a role for S10 phosphorylation in Merlin-mediated contact inhibition. These data suggest that aPKC phosphorylation of Merlin at S12 and S13 could regulate the role of Merlin in contact inhibition and AJ-mediated adhesion.

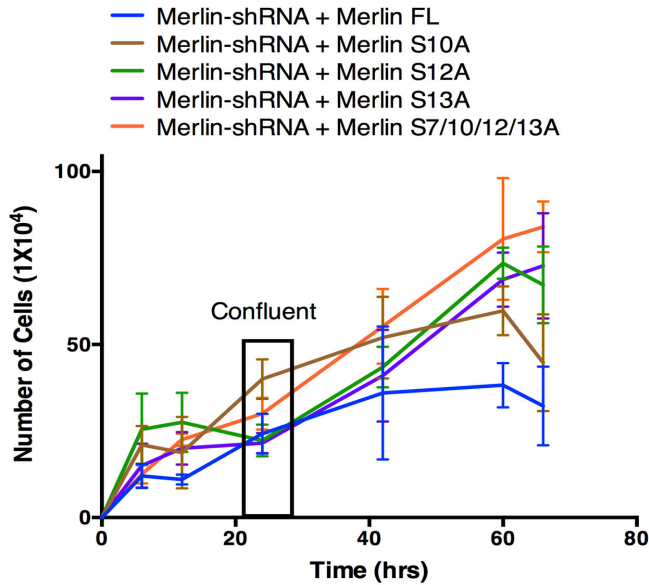
## Discussion

The *Nf2 tumor suppressor gene*-encoded protein, Merlin, has been implicated in the establishment and maintenance of epithelial junctions<sup>84,94</sup> much like the closely related ERM (ezrin, radixin, moesin) protein family. However, the mechanism that regulates Merlin coordination of epithelial junction development is unclear. Herein we described a role for aPKC phosphorylation of the N-terminal region of Merlin that regulates its role in cell adhesion and polarity. To examine the function of Merlin in adhesion and polarity, we utilized the polarized epithelial cell line, Caco-2, due to the potential dynamic nature of Merlin

**A.**



**B.**



**Figure 25. Post-translational modification of the N-terminus of Merlin regulates its role in contact inhibition.** **A)** Growth curve assay of scr (blue), Merlin-shRNA (red) and Merlin full-length reexpression (pink). Full-length reexpression rescues the loss of contact inhibition observed in Merlin knockdown cells. **B)** Growth curve for Merlin point mutants at S10 (brown), S12 (green), S13 (purple) and S7/10/12/13 (orange). Loss of contact inhibition is observed when all four serines are unable to be phosphorylated. Point mutations at S12 and S13 also lose contact inhibition, suggesting a critical phosphorylation by aPKC in regulating Merlin-mediated adhesion.

phosphorylation by aPKC. We found that Merlin knockdown alters actin organization at the cell membrane. This evidence is in line with previous reports that endogenous Merlin decorates the cortical actin cytoskeleton<sup>98</sup> and it is required to link  $\alpha$ -catenin to Par3<sup>84</sup>. Here we show that aPKC phosphorylates the extreme N-terminal region, the first 17 amino acids, of Merlin that is required for its association with  $\alpha$ -catenin at the AJ during junction development<sup>84</sup>.

We have identified a multi-step process in junctional development controlled by aPKC phosphorylation of the N-terminal region of Merlin. The first step elicits proper loading of the junctional polarity complex ( $\alpha$ -catenin/Merlin/Par3) onto the AJ. When phosphorylation of Merlin is inhibited at S13, actin is disorganized suggesting compromised junction integrity. Merlin phosphorylation at residues S7, S10 and S12 does not affect actin dynamics and therefore suggest that these phosphorylation sites are not required for the establishment of AJ adhesions. A second step involving release of Par3/aPKC to the apical domain for formation of functional TJs possibly occurs by phosphorylation of Merlin at residue S7 and/or S12 by aPKC. This is identified through the inhibition of formation of functional TJs in cells where Merlin cannot be phosphorylated at residue S7 and S12. This provides new evidence for a step-wise process of Merlin phosphorylation since actin is organized, but functional TJ development is impaired when Merlin S7 and S12 phosphorylation is blocked. Thus, it is possible that multiple phosphorylation events must occur for release of aPKC/Par3 to the apical membrane to establish TJs. Reduced TER in cells where Merlin cannot be phosphorylated at residue S13 can be attributed to the observation that these cells do not form functional AJs. However, we cannot rule out any additional phosphorylation events that might be required for

Merlin loading onto the AJ or release of aPKC/Par3 to the apical membrane.

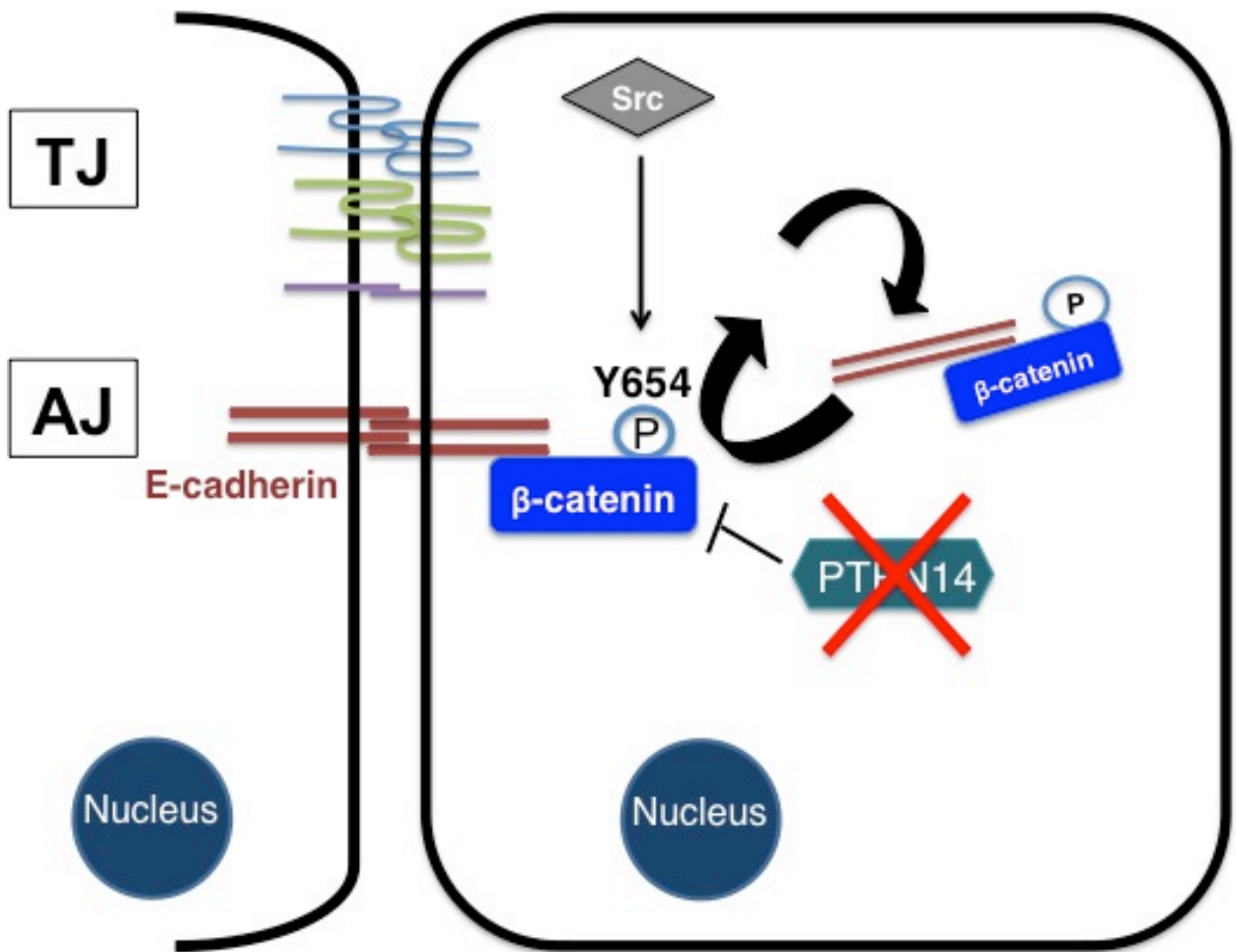
Phosphorylation of Merlin S518 by either PKA or PAK activates Merlin in an open conformation and leads to subsequent Merlin S10 phosphorylation that targets it for proteasomal-mediated degradation<sup>252</sup>. Ablation of Merlin S10 phosphorylation is shown to indirectly alter F-actin organization in fibroblasts<sup>267</sup>. However, we did not see any alteration of the actin cytoskeleton or loss of functional TJs in confluent epithelial cells where Merlin could not be phosphorylated at residue S10 suggesting an alternative mechanism of Merlin cytoskeletal functions. However, in the context of our research, we do not know the phosphorylation state of Merlin S518. Perhaps S518 phosphorylation is only required for Merlin S10 phosphorylation and not for additional phosphorylation events at the N-terminal region of Merlin. This could mean that Merlin phosphorylation at S13 is important for loading Merlin onto the AJ independent of S518 phosphorylation or dependent on an unknown phosphorylation that leaves Merlin in a stable confirmation that is not fully open or closed. This phosphorylation event is followed by an additional phosphorylation of Merlin by aPKC at S7 and/or S12 that releases Par3 and aPKC to the apical membrane for the establishment of functional TJs. Not only are these events critical for junction development and stable cell adhesive contacts, but also for establishing specialized apical and basolateral membranes to obtain polarized epithelia.

**CHAPTER 6.**  
CONCLUSIONS AND FUTURE DIRECTIONS



My dissertation work uncovers novel roles for the FERM domain proteins, PTPN14 and Merlin, in regulating cell adhesion, polarity and lumen formation. FERM domains exist in a variety of mammalian proteins, with the central function of the domain acting as a linker between the plasma membrane and cytoskeletal structures at distinct cellular locations<sup>63</sup>. Both PTPN14 and Merlin exhibit membrane localization in cultured epithelial cells, but the endogenous function of PTPN14 remains elusive and the mechanism of Merlin coordination of epithelial junction development is unclear. Our new data contributes to these gaps in knowledge increasing our understanding of AJ-mediated adhesion and identifying potential future therapeutic avenues in multiple cancers types.

The non-receptor tyrosine phosphatase, PTPN14, was first discovered in 1995 as a FERM protein family member<sup>64</sup> and years later the crystal structure was elucidated to uncover unique structural features of PTPN14 among the PTP family members<sup>268</sup>. In recent years, there has been an explosion of literature focusing on potential roles of PTPN14 in organ development<sup>65</sup>, adhesion<sup>269,270</sup>, motility<sup>67</sup>, proliferation<sup>71,163</sup> and cancer<sup>68,271,272</sup>. However, the endogenous functions of PTPN14 still remain a mystery aside from the fact that it harbors phosphatase activity. One group found that the AJ protein,  $\beta$ -catenin, is a substrate of PTPN14 *in vitro*<sup>67</sup>, but the tyrosine residue was left unidentified. In addition, there is no evidence of any opposing kinase(s) of PTPN14. As modeled in Figure 26, we discovered that PTPN14 opposes Src kinase activity at  $\beta$ -cateninY654 to precisely regulate AJ-mediated adhesion and *ex vivo* lumen formation. PTPN14 activity is necessary for the formation of functional AJs and TJs, a required feature for a cell to possess proper apicobasal polarity. In cells with established epithelial junctions, decreased PTPN14 activity increases E-cadherin membrane movement thereby weakening the AJ. Curiously, the stabilization of E-cadherin in the primordial lumen



**Figure 26. PTPN14 is a major regulator of AJ-mediated adhesion.** In established epithelia, PTPN14 opposes Src-mediated β-catenin-Y654 phosphorylation to regulate AJ adhesion. Our data demonstrates that decreased PTPN14 increases phosphorylation of β-catenin-Y654 and E-cadherin membrane movement potentially as a complex with β-catenin. Destabilization of E-cadherin in the absence of PTPN14 causes cells to lose functional AJs and TJs to ultimately undergo a transition to acquire mesenchymal characteristics.

of three-dimensional intestinal cysts by PTPN14 is necessary for the formation of a single central lumen. We have identified an E-cadherin bridge between two cells prior to primordial lumen formation that is abolished when cells are depleted of PTPN14. Consequently, when adhesion is altered at an early stage, cells cannot form functional AJs and TJs, thereby never establishing apicobasal polarity that is required for lumen morphogenesis. Although this has never been described before, it is interesting to speculate that E-cadherin accumulation at either end of this “bridge” would be controlling the mechanical forces required to open up the luminal space. To date, the inability to form a lumen is seen in only one other recorded incidence, when Ezrin function is eliminated by the expression of a dominant-negative version of Ezrin (Ezrin<sup>Δact</sup>) that cannot bind to nor stabilize actin<sup>94</sup>. This was attributed to the lack of apical integrity mediated by Ezrin, but we also know that apicobasal polarity is regulated by the fence function of the TJ. Prior reports do identify a role for TJ proteins in the increase of internal fluid pressure to facilitate luminal expansion<sup>273,274</sup>. However, when TJs are compromised, the observed outcome is a formation of multiple lumens, which indeed is a feature of loss of cell polarity due to mitotic spindle misorientation<sup>84</sup>. Therefore, our data demonstrates that the underlying reason for a no lumen phenotype is distinct from that of a multiple lumen. We have shown that adhesion is tightly regulated by the AJ during the early stages of primordial lumen formation and since the formation of functional TJs is dependent on functional AJs, PTPN14 knockdown cells have chronic weakening of AJs and never form a lumen in 3D. This is the first evidence highlighting a critical role of PTPN14 and AJ-mediated adhesion in lumen morphogenesis.

Observations that PTPN14 is mutated or levels are altered in colorectal, breast, and pancreatic cancer suggest that PTPN14 may be important for the maintenance of epithelial tissues. The exact threshold at which a decrease or increase in PTPN14 activity alters cell adhesion is yet to be determined. There is evidence to suggest that PTPN14 has oncogenic

potential in endothelial<sup>163</sup> and epithelial cells<sup>65,270</sup>. However, there is also data demonstrating that PTPN14 can act as a tumor suppressor<sup>65,66,72,206,220,271,272</sup>, suggesting that the level of PTPN14 activity is critical to maintain the balance of cellular homeostasis. We demonstrate that over time, decrease of PTPN14 activity primes cells to undergo an EMT transcriptional response, complete with repression of E-cadherin possibly through Snail2/Slug, loss of collective cell migration and loss of contact inhibition of proliferation. At this time, we cannot rule out alterations of any additional proteins that may contribute to the mesenchymal characteristics of these cells. However, we have established that EMT observed in PTPN14 knockdown cells is independent of the TGF $\beta$  and Hippo signaling pathways. Additionally, Notch signaling is activated in cells with decreased PTPN14 and Notch was previously shown to directly bind and activate the Snail2/Slug promoter<sup>217</sup>. Both TGF $\beta$  and Hippo are implicated in regulating EMT in multiple cell types coupled with either an increase or decrease in PTPN14 activity<sup>65,71</sup>. We have not observed any transcriptional effects on TGF $\beta$  or Yap pathway targets in our work in intestinal epithelial cells. Interestingly, PTPN14 is shown to translocate to the nucleus in proliferating vascular endothelial cells<sup>163</sup>, but we have been unable to observe nuclear PTPN14 in semi-confluent or confluent monolayers of epithelial cells. This could suggest that PTPN14 may have precise tissue-specific roles that could be modulated by other pathways acting upon PTPN14 transcription or post-translational regulation of PTPN14 by other proteins. We have yet to learn what protein(s) regulate PTPN14 activity and expression, which might provide insight into how PTPN14 alters cell adhesion to initiate or accelerate disease progression.

Two chapters of my dissertation highlight the significance of PTPN14, a protein that is relatively poorly understood in terms of endogenous function and regulation. Our work has uncovered novel roles for PTPN14 in regulating AJ-mediated adhesion through stabilization of E-cadherin/ $\beta$ -catenin. Over time the reduced activity of PTPN14 causes an EMT response in

cultured cells. Our potential future research for PTPN14 could unfold in multiple ways including; 1) investigating the fine-tuning regulation of PTPN14 phosphatase activity at the AJ and 2) the big picture impact of PTPN14 loss or reduced activity has on disease initiation and/or progression.

### **Fine-tuning PTPN14 activity at the AJ**

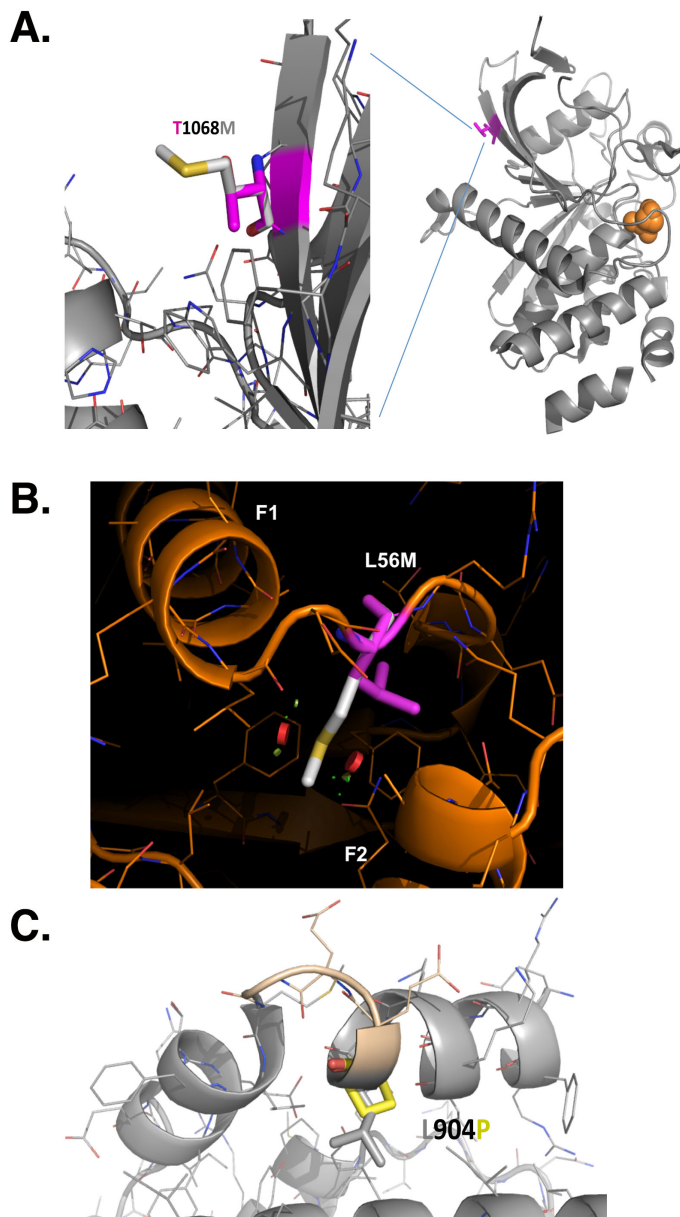
Although we now have a better understanding of a mechanism that is controlled by PTPN14 activity at the AJ, questions remain on what proteins might be regulating PTPN14. During lumen morphogenesis, what triggers PTPN14 to dephosphorylate  $\beta$ -catenin Y654 thereby stabilizing the primordial cell contact to facilitate the formation of a single lumen? Since tissue morphogenesis is contingent on collective cell movements<sup>275-280</sup> and mechanical cues<sup>281-285</sup>, perhaps there are mechanical forces at play that intrinsically signal to activate PTPN14. Studies in *C. elegans* identify a need for continuous Notch signaling, which controls the Rho kinase LET-501 to induce actomyosin-mediated contraction of the apical lumen in the developing vulval tube<sup>286</sup>. It is possible that PTPN14 may be responding to mechanical forces set in motion by Notch signaling or vice versa. Future work must be done to gain a firm understanding of exactly what mechanical forces drive the opening of the primordial cell contact, the potential critical first step to forming a single lumen.

Conversely, what is regulating PTPN14 activity in established epithelia? One could hypothesize that there is a built-in feedback loop between PTPN14 and its opposing kinase, Src, during the maintenance of established epithelial adhesion. Increased growth-factor dependent signaling of Src reduces adhesion<sup>159</sup>, but how PTPN14 responds to this increase in Src is unknown. Also of interest is whether or not PTPN14 is cooperating with additional AJ phosphorylation events in the establishment and maintenance of epithelial adhesion in tissue

homeostasis. Does PTPN14 reside at the AJ permanently or is it a transient component that is only recruited to the AJ to dephosphorylate  $\beta$ -catenin and possibly other AJ proteins under certain conditions? Two examples of AJ proteins that are regulated by tyrosine phosphorylation to stabilize the AJ are E-cadherin and p120-catenin. E-cadherin tyrosine phosphorylation at residues 753-755 by the Src kinase facilitates ubiquitination and subsequent docking of the E3-ligase Hakai<sup>287</sup>. In turn, this ubiquitination leads to internalization of E-cadherin and disruption of the AJ<sup>288</sup>. It would be interesting to examine the phosphorylation state of E-cadherin Y733-735 in Caco-2 cells and compare it to what we observe with regards to PTPN14 mediated AJ stabilization to obtain a better picture to how Src activity is opposed at the AJ. Src also phosphorylates p120-catenin on eight tyrosine residues of its N-terminal region and this event serves as recruitment for interacting proteins carrying SH2 domains<sup>289,290</sup>. There are instances where p120-catenin phosphorylation can increase its binding affinity to cadherin although this increase is not always observed<sup>291</sup>. This is important considering that p120 can protect E-cadherin from internalization, which has significant impacts on AJ dynamics<sup>158</sup>. These are a few examples that provide strong evidence that phosphorylation switches play an important role in the stabilization of the AJ, but common biochemical techniques can limit the scope of phosphorylation events at the AJ. Phospho-proteomic data have identified multiple phosphorylated serine/threonine and tyrosine residues of AJ proteins<sup>292</sup>. This approach could serve as a first step to help increase our knowledge of phosphorylation switches that regulate the AJ. A future challenge will be to take a comprehensive look at the phosphorylation network at the AJ with other regulatory switches to gather a complete understanding of how signaling and force regulates AJ dynamics.

## **PTPN14 in disease initiation and progression**

We are particularly interested in the function(s) of PTPN14 in pathological conditions such as CRC. We are beginning to work with CRC patient-derived mutations in our cell culture model to understand if the mutations have similar effects on E-cadherin membrane mobility as reduced PTPN14 activity. Wang, *et al* discovered a distribution of PTPN14 mutations in CRC patients with multiple mutations uncovered in the non-conserved interlinking region, a single mutation in the FERM domain and a single mutation in the PTP domain<sup>66</sup>. The two mutations found in the FERM (L56M) and PTP (T1068M) domains are of interest to us since these are conserved regions of the PTPN14 protein. With the help of Dr. Stefan Arold (King Abdullah University of Science and Technology), we modeled these CRC patient-derived mutations to understand the potential biological ramifications they may have on protein structure and function. Possible effects of the CRC mutation T1068M (Figure 27A) have been assessed previously as having no potential effect on protein function since this residue sticks out into solvent on the opposite face from the active phosphatase site at C1121<sup>268</sup>. As such, we do not anticipate that this mutation has any detrimental effects on protein structure or function. However, the story is quite different for the CRC patient-derived mutation in the FERM domain at L56M. From the structural modeling, we have hypothesized that the L56M mutation in the FERM domain has the potential to interfere with possible binding partners due to the nature of a the mutated proline residue sticking out into the binding pocket between the F1 and F2 lobes of the FERM domain (Figure 27B). Through bioinformatics approaches, with our collaborator, Dr. Bin Liu (MD Anderson Cancer Center), we have discovered an additional CRC patient-derived mutation at L904P that could lead to a local or even global destabilization of the PTP domain (Figure 27C). A future challenge is to determine if these mutations cause weakening of AJ-mediated adhesion similar to decreased PTPN14 and if they increase signaling pathways, such as Notch, that are



**Figure 27. Structural modeling of PTPN14 CRC patient-derived mutations.** A) The PTP domain mutation T1068M sticks out into solvent suggesting no potential effect on protein structure and stability. Wild-type residues are colored with carbons in pink and mutant carbons are in grey. B) FERM domain mutation L56M sticks out into a potential binding pocket between the F1 and F2 lobes of the FERM domain presenting a potential inhibition of binding partners. Wild-type residues are colored with carbons in pink and mutant carbons are in white. C) The L904P mutation is situated at the C-terminal end of the first  $\alpha$  helix ( $\alpha$ 1) of the PTP domain. This mutation may produce strain on the loop region and C terminal end of  $\alpha$ 1 leading to potential destabilization of the PTP domain. Wild-type residues are colored with carbons in grey and mutant carbons are in yellow. Modeling courtesy of Dr. Stefan Arold (KAUST) key regulators of disease initiation and/or progression.

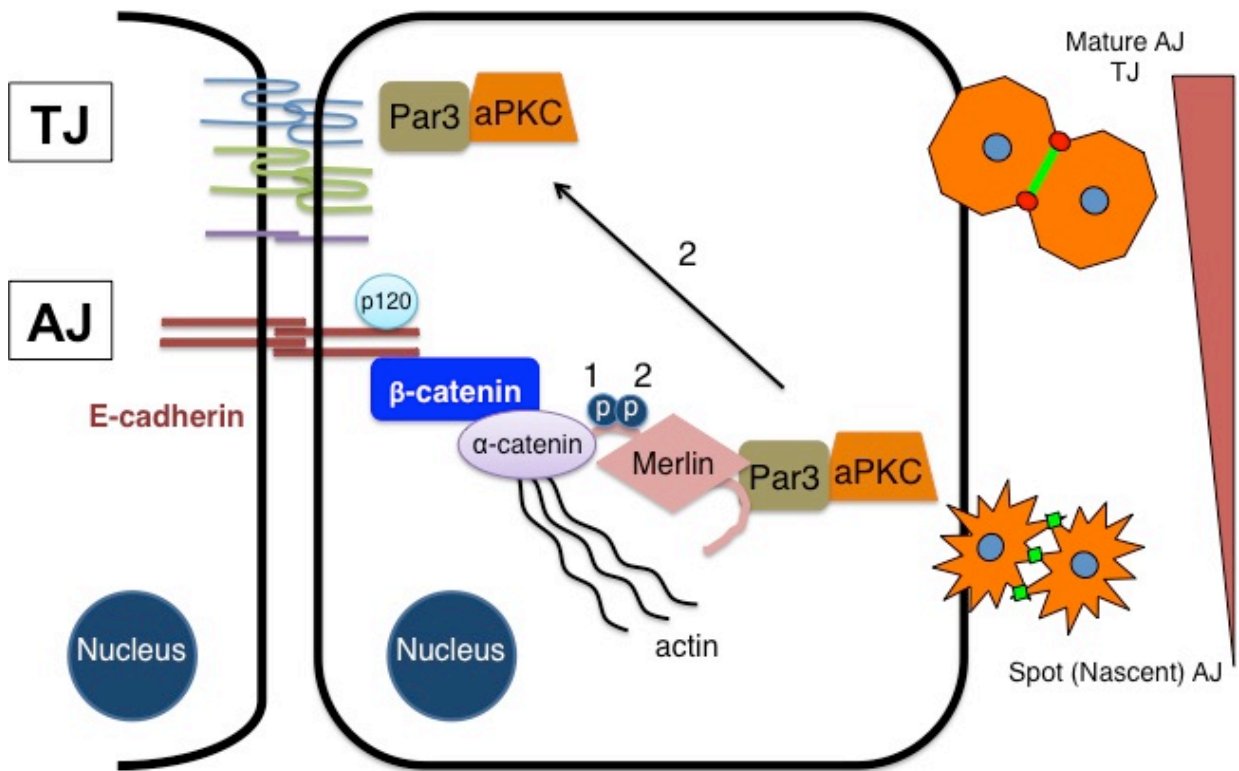


Ultimately, the desire is to study the impact of PTPN14 loss in human disease. Unfortunately at this time there is no PTPN14 conditional knockout mouse models in existence. To this end, a potential future research endeavor of this project is to create a mouse model to study PTPN14 function *in vivo*. We hypothesize that if a complete knockout mouse of PTPN14 is not embryonic lethal, then these mice will possibly present with development defects such as decreased barrier function of the skin and abnormalities in organs that form tubes such as the intestine, kidney and mammary gland. This hypothesis takes into consideration the importance of PTPN14 in establishing adhesive contacts in the early stages of lumen morphogenesis. There is a possibility that PTPN14 knockout mice could have defects, but ones that are not life threatening. If this is the case, a conditional knockout of PTPN14 could be utilized to selectively target tissues that have high expression of PTPN14, such as brain, cardiac and intestinal tissues. The hypothesis would then be that adhesive contacts would weaken and gradually cause sensitization of cells to undergo EMT, similar to what we have observed in our cell culture model. Another viable option would be a xenograft model where severely compromised immunodeficient (SCID) mice are injected with PTPN14-deficient cells and ask whether metastasis occurs. Dasatinib treatment could also be employed in this model to identify if the response is similar to what is seen in cultured cells. In addition, this system would allow a tool to express our patient-derived mutations to determine if these mutations affect the invasive potential of the cells. These data could provide substantial evidence for the function of PTPN14 *in vivo* and for the role it has in regulating tumor initiation and/or progression.

## **Merlin: Adhesion, polarity and tumor progression**

The *Nf2*-encoded protein, Merlin is a classical tumor suppressor that when mutated or inactivated leads to familial schwannomas and meningiomas<sup>293</sup>. Although not as prevalent, mutations causing loss of *NF2* function in non-nervous system cancers have been reported, such as CRC<sup>294</sup>, mesotheliomas<sup>78,295,296</sup>, prostate cancer<sup>297</sup>, melanoma and thyroid cancer<sup>298</sup>. Since there is not a high prevalence of *Nf2* mutations in non-nervous cells it raises the possibility that Merlin can be inactivated at the post-translational level. It is well documented that Merlin can be post-translationally modified, but how this affects its roles in regulating contact-dependent inhibition of proliferation, cell polarity and adhesion is poorly understood. Merlin is necessary to bridge the AJ protein,  $\alpha$ -catenin, to the apical protein, Par3, for the establishment of functional junctions in epithelial adhesive contacts<sup>84</sup>. Furthermore, the extreme N-terminal region of Merlin is required for this interaction. Consequently, loss of Merlin or lack of its N-terminal region impedes the formation of functional TJs and proper apicobasal polarity.

As modeled in Figure 28, we discovered that aPKC phosphorylates serine sites in the unique N-terminal region of Merlin in a step-wise fashion to regulate its function in establishing junction-mediated adhesion and polarity. The first step includes loading of the junctional polarity complex onto the AJ followed by a second phosphorylation to release Par3 from to AJ to the apical membrane for the formation of TJs. Phosphorylation of Merlin S13 promotes actin organization, formation of functional TJs and Merlin localization at the membrane. This is the first potential step that is required for proper loading of Merlin onto the AJ during the development of functional junctions, which is why we see impaired TJs since TJ formation is dependent on the establishment of functional AJs. Phosphorylation of Merlin S7 and S10 are also required for the formation of functional TJs, but are not essential for the organization of actin and Merlin localization at the membrane, making them likely candidates as phosphorylation sites



**Figure 28. Post-translational modification of the N-terminal region of Merlin by aPKC regulates its function in the establishment of junctions and apicobasal polarity.** Our data provides evidence of a multi-step process in junctional development controlled by aPKC phosphorylation of the N-terminal region of Merlin. An initial phosphorylation at S13 (1) is required to load the junctional polarity complex ( $\alpha$ -catenin/Merlin/Par3) onto the AJ. A second phosphorylation event (2) possibly at S7 and/or S12 releases Par3/aPKC to the apical domain for the formation of functional TJs. Phosphorylation of the N-terminal region of Merlin by aPKC is necessary for Merlin localization at the membrane, actin organization, the establishment of functional TJs and the role of Merlin in contact-dependent inhibition of proliferation.

required for localization of Par3 to the apical membrane to facilitate TJ formation. However, we cannot be certain that these are the only phosphorylation events required for Merlin function in regulating junctional development. The FERM domain and C-terminal tail of Merlin can associate to change the accessibility to its binding partners<sup>61</sup>. Early reports described that phosphorylation of Merlin at S518 is shown to open the protein thereby leading to its inactivation<sup>299,300</sup>. Later this was shown to be a static process whereby Merlin is not fully open or closed, but tends to vary in degrees of openness<sup>301,302</sup>. This suggests that S518 phosphorylation has the ability to increase the interaction of the FERM and C-terminal tail of Merlin to leave it in a fairly closed and inactive state away from the cell membrane<sup>302</sup>. Membrane localization of Merlin is only lost when Merlin cannot be phosphorylated at S13, suggesting that S518 phosphorylation may not act in concert with additional phosphorylation events occurring during junctional development. To test this, we could express the Merlin S7 and S13 non-phosphorylatable mutants and perform Western analysis at different time points in junctional development and observe whether the phosphorylation state of Merlin-S518 is altered. Nevertheless, we do see that phosphorylation of the N-terminal region of Merlin has an effect on growth control and contact-dependent inhibition of proliferation, a hallmark of tumorigenesis.

Acquisition of our functional data has led us to look specifically at *in vitro* phosphorylation of Merlin by aPKC. We currently have six GST Merlin protein constructs to use as substrates for aPKC: GST-Merlin, GST-Merlin-S7A, GST-Merlin-S10A, GST-Merlin-S12A, GST-Merlin-S13A and GST-Merlin-Δ1-17. Due to recent literature indicating similar functions for the aPKC<sub>1</sub> and aPKC<sub>ζ</sub> isoforms, we are performing these kinase assays to possibly identify isoform-specific functions for aPKC in Merlin phosphorylation. Based on our functional data, it is possible that we will observe multiple sites of Merlin phosphorylation by the different aPKC isoforms. Future experiments could then be geared towards mechanistically showing at which

point in junctional development each phosphorylation event occurs on the N-terminal region of Merlin. This could include co-immunoprecipitation experiments at multiple time points during junctional development similar to what was performed with PTPN14 (See Figure 5). With this, the development of phospho-specific antibodies against each serine site in the N-terminal region of Merlin would be useful for future analysis. Also, we plan to address if Merlin still interacts with polarity proteins when the N-terminal region is unable to be phosphorylated. Preliminary data suggests that phosphorylation of specific serine(s) may be critical for the interaction of Merlin with aPKC and Par3.

Merlin loss causes the formation of multiple lumens in 3D, which can be restored with full length Merlin. Interestingly, our data and other reports show that this rescue of a single lumen is dependent on the N-terminal region of Merlin<sup>94</sup>. We are currently determining if any of our candidate serine sites in this N-terminal region must be phosphorylated to ensure the formation of a single lumen. From our data, we hypothesize that S7 or S13 phosphorylation will be required for the formation of a single lumen. Serine 12 may also be important due to the close proximity to S13, although we have yet to see similarities between the two sites. This hypothesis stems from the fact that S7 and S13 phosphorylation are required for the establishment of functional TJs that serve as a fence to divide the apical and basal membranes, a prerequisite for apicobasal polarity and ultimately, lumen morphogenesis.

Furthermore, we are interested in identifying the role of the Merlin/aPKC/Par3 polarity complex in tumor progression. Loss of Merlin in cultured cells leads to contact-dependent inhibition of proliferation, properties that are utilized to differentiate between normal and cancerous cells. Since loss of Merlin in epithelial cells alter Par3 and aPKC localization, we are interested in examining the pathway that governs cell polarity to determine if these proteins act in concert with Merlin to facilitate proper epithelial cell polarity and thereby avoid tumorigenic

behavior. Specifically, we are interested in understanding the role of the Merlin/Par3/aPKC polarity complex in the regulation of *Nf2* tumors. We seek to understand if localization of Par3 and aPKC is altered in *Nf2* tumor cells and tissues to determine their roles in tumor progression. We have both human and mouse *Nf2*-deficient schwannoma tumor cells (HEI193 and SC4 cells) where we can express full-length Merlin via lentiviral infection into these Schwann cells to determine if this relocalizes the Par3/aPKC complex. We hypothesize that Par3 and/or aPKC will not localize to proper membrane compartments in *Nf2*-deficient cells. This result would correspond with what we have observed in epithelial tissues and indicate that membrane localization of Par3 and aPKC is dependent on Merlin. If one of the N-terminal serine Merlin point mutants blocks binding to either Par3 or aPKC, we could use that mutant to determine if re-establishment of polarity in *Nf2* mutant Schwann cells can restore Schwann cell function. Alternatively, Par3 and aPKC could localize to their corresponding membrane compartments in *Nf2*-deficient cells, suggesting that the function of these proteins in *Nf2* tumor cells is not dependent on mis-localization, but instead occurs through other functions.

This work uncovers novel roles for the FERM proteins, PTPN14 and Merlin, in regulating epithelial cell adhesion and apicobasal polarity. We highlight the importance of PTPN14 in 3D lumen morphogenesis, regulating the stability of the AJ protein, E-cadherin. E-cadherin is a major component of the AJ that is required for stable cell adhesive contacts. E-cadherin forma a bridge at the primordial contact that is dependent on PTPN14 and the phosphorylation state of  $\beta$ -cateninY654. As the primordial lumen opens to reveal an apical membrane facing the inner luminal surface, E-cadherin clusters at either end of the primordial contact as if it is needed to mechanically push open the luminal space and maintain cell adhesion. Although not fully understood, this process is dependent on PTPN14 activity; otherwise cells fail to form a lumen in 3D. This makes PTPN14 a significant contributor to the development of *ex*

*vivo* lumen morphogenesis. Conversely, Merlin is required to establish apical identity following the initial primordial cell contact. This is done so through phosphorylation of the N-terminal region of Merlin by aPKC. Two phosphorylation events could occur to load Merlin onto the AJ and release Par3 and aPKC from the AJ to the TJ. If these two events do not occur then polarity is disrupted and cells will form a multiple lumen in 3D. The difference in luminal phenotypes with decreased PTPN14 and Merlin highlight the importance of the early establishment of cell adhesion and the coordination of apicobasal polarity, respectively. This model provides insight into how the formation of biological tubes is regulated during normal development.

Also, we have identified that PTPN14 and Merlin are required for maintaining adhesion in established epithelia. A balance of PTPN14 activity is necessary for the maintenance of adhesion through dephosphorylation of  $\beta$ -cateninY654. PTPN14 and Merlin depletion leads to the loss of functional junctions, which is indicative of loss of barrier function. Defects in epithelial barrier function are observed in chronic conditions that can lead to intestinal inflammatory disease and colitis-associated cancers<sup>129</sup>. Importantly, over time decreased PTPN14 causes cells to undergo a transition to a mesenchymal phenotype indicating that prolonged reduced activity of PTPN14 can initiate tumor progression. This makes PTPN14 of clinical relevance as a potential drug target for inflammatory diseases of the intestines and intestinal cancers.

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